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<b>(54) Title:</b> SUBSTRATE TRAPPING PROTEIN TYROSINE PHOSPHATASES  <b>(57) Abstract</b>  Novel protein tyrosine phosphatases in which the invariant aspartate residue is replaced with an alanine residue and which bind to a tyrosine phosphorylated substrate and are catalytically attenuated are described. Also described are methods of identifying tyrosine phosphorylated proteins which complex with the described protein tyrosine phosphatases.		

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SUBSTRATE TRAPPING PROTEIN TYROSINE PHOSPHATASESBackground of the Invention

The protein tyrosine phosphatase (PTP) family of enzymes consists of more than 500 structurally diverse proteins which have in common the highly conserved 250 amino acid PTP catalytic domain, but which display considerable variation in their non-catalytic segments (Charbonneau and Tonks, Annu. Rev. Cell Biol. 8:463-493 (1992); Tonks, Semin. Cell Biol. 4:373-453 (1993)). This structural diversity presumably reflects the diversity of physiological roles of individual PTP family members, which in certain cases have been demonstrated to have specific functions in growth, development and differentiation (Desai et al., Cell 84:599-609 (1996); Kishihara et al., Cell 74:143-156 (1993); Perkins et al., Cell 70:225-236 (1992); Pingel and Thomas, Cell 58:1055-1065 (1989); Schultz et al., Cell 73:1445-1454 (1993)). Although recent studies have also generated considerable information regarding the structure, expression and regulation of PTPs, the nature of the tyrosine phosphorylated substrates through which the PTPs exert their effects remains to be determined. Studies with a limited number of synthetic phosphopeptide substrates have demonstrated some differences in substrate selectivity of different PTPs (Cho et al., Protein Sci. 2: 977-984 (1993); Dechert et al., Eur. J. Biochem. 231:673-681 (1995)), and have indicated preferences for certain amino acid residues at particular positions around the phosphorylated tyrosine residue (Ruzzene et al., Eur. J. Biochem. 211:289-295 (1993); Zhang et al., Biochemistry 33:2285-2290 (1994)). This indicates that PTPs display a certain level of substrate selectivity in vitro, although

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the physiological relevance of the substrates used in these studies is unclear.

#### Summary of the Invention

As described herein, the substrate specificity of  
5 mammalian protein tyrosine phosphatases (PTPs) has been  
investigated using a novel substrate trapping approach in  
which mutant or altered forms of the mammalian PTP, also  
referred to as substrate trapping PTPs, are used to bind  
(trap) one or more substrates of the PTP. Binding of the  
10 substrate trapping PTP with a substrate of the PTP results  
in the formation of a complex which can be readily  
observed, and, if desired, isolated, and characterized.  
The mutant forms of the PTPs have attenuated catalytic  
activity (lack catalytic activity or have reduced catalytic  
15 activity) relative to the wild type PTP but retain the  
ability to bind tyrosine phosphorylated substrate(s) of the  
wild type PTP.

The methods of the present invention are specifically  
exemplified herein with respect to the phosphatases PTP1B  
20 and PTP-PEST; however, it is understood that the invention  
is not limited to these specific PTPs but is applicable to  
all members of the PTP family. In order to identify  
potential substrates of PTP1B and PTP-PEST, mutant (i.e.,  
altered or substrate trapping) forms of PTP1B and PTP-PEST  
25 were generated which were catalytically attenuated but  
retained the ability to bind substrates. These mutant PTPs  
associated in stable complexes with proteins which were  
identified by immunoblotting as p210 bcr:abl and p130<sup>cas</sup>,  
respectively. These associations were observed in lysates  
30 from several cell lines and in transfected COS cells,  
indicating that p210 bcr:abl and p130<sup>cas</sup> represent major  
physiologically relevant substrates for PTP1B and PTP-PEST.

These results provide the first demonstration of PTPs  
having inherently restricted substrate specificity in vivo.

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The methods used to identify p210 bcr:abl and p130<sup>cas</sup> as specific substrates for PTP1B and PTP-PEST, respectively, are generally applicable to any member of the PTP family, of which approximately 500 members have currently been reported, and can be used to determine the physiological substrates of other members of the PTP family.

One embodiment of the invention relates to novel mutant PTPs in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ). These PTPs retain the ability to form a complex with, or bind, their tyrosine phosphorylated substrates, but are catalytically attenuated. In one embodiment, the invention relates to the phosphatase PTP1B in which the invariant aspartate residue at position 181 is replaced with alanine (D181A). In another embodiment the invention relates to the phosphatase PTP-PEST in which the invariant aspartate residue at position 199 is replaced with an alanine (D199A). Another embodiment of the invention relates to a PTP-PEST phosphatase in which the cysteine residue at position 231 is replaced with a serine (C231S). The invention also relates to other mutant or substrate trapping PTPs in which the invariant aspartate residue is replaced with or changed to another amino acid residue, such as alanine. The invariant aspartate residue can be identified in other PTPs by aligning the PTP nucleotide sequence with the nucleotide sequence of a PTP for which the location of the invariant aspartate residue is known.

The invention also relates to a method of identifying a tyrosine phosphorylated substrate of a protein tyrosine phosphatase. According to one embodiment of the present invention, a tyrosine phosphorylated protein of interest is combined with one or more PTP(s) in which the invariant aspartate residue is replaced with an amino acid which does

not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ), and the presence or absence of a complex between the protein and the PTP(s) is determined.

- 5 Presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the PTP. The PTP DA mutant binds to or complexes with its substrate but does not dephosphorylate it (or does so very slowly), thereby allowing the complex to be observed and, optionally,  
10 isolated and identified. In a particular embodiment of the invention, the invariant aspartate is replaced with an alanine residue (a PTP DA mutation or alteration)

- In an alternative embodiment of the present invention, a PTP of interest in which the invariant aspartate residue  
15 is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ), is combined with one or more tyrosine phosphorylated proteins, and the presence or absence of a  
20 complex between the protein(s) and the PTP is determined. Presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the PTP. The PTP DA mutant binds to or complexes with its substrate but does not dephosphorylate it (or does so very slowly),  
25 thereby allowing the complex to be observed, and, optionally, isolated and identified. In one embodiment of the invention, the invariant aspartate residue is replaced with an alanine residue (a PTP DA mutation or alteration)

- The present invention also relates to a method of  
30 identifying a tyrosine phosphorylated substrate of a protein tyrosine phosphatase wherein more than one tyrosine phosphorylated protein of interest is combined with more than one PTP of interest in which the invariant aspartate residue is replaced with an amino acid which does not cause  
35 significant alteration of the  $K_m$  of the enzyme but which

results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ) (e.g., the invariant aspartate is replaced with an alanine residue). Complexes formed in the combination can be isolated and the component PTP and

5 substrate can be identified.

The invention also pertains to a method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a mammal a PTP in which the invariant aspartate residue is replaced with an amino acid

10 which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ) (e.g., the invariant aspartate is replaced with an alanine residue) and which forms a complex with the tyrosine phosphorylated protein.

15 The PTP mutant binds to the phosphorylated protein without dephosphorylating it, thereby inhibiting the activity of the protein and reducing its downstream effects.

For example, the invention relates to a method of reducing the transforming effects of oncogenes associated with  $p130^{cas}$ , a substrate of PTP-PEST, comprising

20 administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an alanine residue. Wild type PTP-PEST binds and dephosphorylates  $p130^{cas}$ , thereby negatively regulating its downstream effects. DA mutants of PTP-PEST bind but do not dephosphorylate  $p130^{cas}$  (or dephosphorylate it at a reduced rate); the substrate is thus tied up in the complex with the substrate trapping form of PTP-PEST and cannot exert its downstream effects. Similarly, the invention relates to a

25 method of reducing the formation of signalling complexes associated with  $p130^{cas}$ , particularly those signalling complexes which induce mitogenic pathways, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an

30 alanine residue.

The present invention also relates to assays for identifying agents which alter, e.g., enhance or inhibit, the interaction between a PTP and its phosphorylated substrate. Agents identified by these assays can be  
5 agonists (e.g., agents which enhance or increase the activity of the PTP) or antagonists (e.g., agents which inhibit or decrease the activity of the PTP) of PTP activity. The agent may be an endogenous physiological substance or may be a natural or synthetic drug, including  
10 small organic molecules.

For example, the tyrosine phosphorylated substrate of a PTP can be identified by the methods described herein. An enzymatic activity assay utilizing the wild type PTP can be carried out in the presence of an agent to be tested, and  
15 the resulting amount of enzyme activity can be compared with the amount of enzyme activity in the absence of the agent to be tested. A decrease in the enzymatic activity in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate.  
20 Conversely, an increase in the enzymatic activity in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Alternatively, a competitive binding assay can be carried out utilizing the mutant PTP in the presence of an  
25 agent to be tested, and the resulting extent of binding of the mutant PTP to its substrate can be compared with the extent of binding in the absence of the agent to be tested. A decrease in the extent of binding in the presence of the agent to be tested indicates that the agent inhibits the  
30 interaction between the PTP and its substrate. Conversely, an increase in the extent of binding in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Thus, the compositions and methods described herein  
35 are useful in identifying the tyrosine phosphorylated



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substrates of members of the PTP family of phosphatases, as well as in regulating the activity of identified substrates. The compositions and methods described herein are also useful for identifying tyrosine phosphorylated proteins which are related to a particular disease or disorder, and to methods of screening for modulators which enhance or inhibit the PTP/substrate interaction for use in therapeutic applications.

#### Brief Description of the Drawings

10        Figures 1A and 1B show a multiple sequence alignment of the catalytic domains of PTPs. In Figure 1A, cytosolic eukaryotic PTPs and domain 1 of RPTs are combined into one group, domains 2 of RPTs are in a second group, and the *Yersinia* PTP is in a third. Invariant residues shared among  
15 all three groups are shown in red. Invariant and highly conserved residues within a group are shown in blue and green, respectively. Within the *Yersinia* PTP sequence, residue that are either invariant or highly conserved between the cytosolic and RPTP domain sequences are colored  
20 blue and green, respectively. The position of residues of PTP1B that interact with the peptide are indicated with a red arrow, and the residue numbering at the bottom of the alignment corresponds to that for PTP1B. Figure 1B is a black and white photocopy of Figure 1A in which the colored  
25 areas are indicated with labeled arrows.

Figure 2 shows the  $V_{max}$  and  $K_m$  of various PTP1B mutants toward RCML.

#### Detailed Description of the Invention

30        The PTP family of enzymes contains a common evolutionarily conserved segment of approximately 250 amino acids known as the PTP catalytic domain. Within this

conserved domain is a unique signature sequence motif, [I/V]HCXAGXXR[S/T]G, that is invariant among all PTPs. The cysteine residue in this motif is invariant in members of the family and is known to be essential for catalysis. It functions as a nucleophile to attack the phosphate moiety of the incoming substrate. If the cysteine residue is altered by site-directed mutagenesis to serine (CS mutants) or alanine (CA mutants), the resulting PTP is catalytically attenuated but retains the ability to complex with, or bind, its substrate, at least in vitro. These results have been confirmed relative to MKP-1, a member of the PTP family (Sun et al., Cell 75:487-493 (1993)), as well as other PTPs. However, although these CS mutants can in general bind effectively to phosphotyrosyl substrates in vitro, in many cases such complexes cannot be isolated in vivo. Thus, the CS mutants are limited in their applicability and cannot be used to isolate all combinations of PTPs and substrates.

The crystal structures of PTP1B alone (Barford, et al., Science 263:1397-1404 (1994)) and in a complex with a phosphotyrosine-containing peptide (Jia et al., Science 268:1754-1758 (1995)) were recently determined. These structures indicated twenty seven invariant residues (Barford et al., 1994), one of which is an aspartate residue. This aspartate residue is invariant across the catalytic domains of PTP family members. That is, if the amino acid sequences of the PTP family members are aligned, the aspartate residue is present in each PTP at a corresponding location, although the position numbers may be different due to the shifts required to maximize alignment (see the Figure (from Barford et al., Nature Struc. Biol. 2:1043-1053 (1995)) for an alignment of various PTP sequences). Sequences for which the alignment has not yet been published can readily be aligned with other known PTP sequences, e.g., utilizing available computer software such as GENEWORKS.

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Thus, mutant PTPs other than those specifically described herein can readily be made by aligning the amino acid sequence of the PTP catalytic domain with those described herein, identifying the invariant aspartate residue, and changing the residue by site-directed mutagenesis. Although the specific examples of PTP mutants described herein are aspartate to alanine mutants (DA mutants), it is understood that the invention is not limited to changes of aspartate to alanine. The invariant aspartate residue can be changed, e.g., by site-directed mutagenesis, to any amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ). For example, the invariant aspartate residue can be changed or mutated to an alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, lysine, arginine or histidine.

As described herein, pervanadate-treated cells were used as an abundant source of tyrosine phosphorylated proteins to investigate the substrate specificity of PTP-PEST. PTP-PEST is an 88 kDa cytosolic PTP (Charest *et al.*, *Biochem. J.* 303:425-432 (1995); den Hertog *et al.*, *Biochem. Biophys. Res. Commun.* 184:1241-1249 (1992); Takekawa *et al.*, *Biochem. Biophys. Res. Commun.* 189:1223-1230 (1992); Yang *et al.*, *J. Biol. Chem.* 268:6622-6628 (1993); Yang *et al.*, *J. Biol. Chem.* 268:17650 (1993)) which is expressed ubiquitously in mammalian tissues (Yi *et al.*, *Blood* 78: 2222-2228 (1991)), and which exhibits high specific activity when assayed *in vitro* using artificial tyrosine phosphorylated substrates (Garton and Tonks, *EMBO J.* 13:3763-3771 (1994)). It has previously been demonstrated that PTP-PEST is subject to regulation via phosphorylation of Ser39 *in vitro* and *in vivo*. This modification is catalyzed by both protein kinase C (PKC) and protein kinase

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A (PKA), and results in reduced enzyme activity as a consequence of an increase in the  $K_m$  of the dephosphorylation reaction (Garton and Tonks, EMBO J. 13:3763-3771 (1994)). It appears likely that further  
5 regulatory mechanisms exist for PTP-PEST, since this enzyme would be expected to exert a considerable negative influence on the tyrosine phosphorylation state of cytosolic substrates of tyrosine kinases. One possibility is that this influence could be limited by the substrate specificity  
10 of PTP-PEST.

The substrate specificity of PTP1B was investigated utilizing the same methods outlined for PTP-PEST, with the exception that the cells were not treated with pervanadate. A combination of in vitro dephosphorylation and substrate  
15 trapping experiments were used to study the substrate interactions of PTP1B and PTP-PEST. The substrate trapping methods outlined herein are generally applicable to any PTP by virtue of the shared invariant aspartate residue, and should therefore prove useful in delineating the substrate  
20 preference of other PTP family members. In particular, the use of mutant, catalytically impaired PTPs to trap, and thereby isolate, potential substrates will greatly facilitate the identification of physiologically important substrates for individual PTPs, leading to improved  
25 understanding of the roles of these enzymes in regulation of cellular processes.

One embodiment of the invention relates to novel PTPs in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of  
30 the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ). These PTPs retain the ability to form a complex with, or bind, their tyrosine phosphorylated substrates but are catalytically attenuated. As defined herein, "attenuated"  
35 activity is intended to mean that the phosphatase retains a

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similar  $K_m$  to that of the wild type phosphatase but has a  $V_{max}$  which is reduced by a factor of at least  $10^4$  relative to the wild type enzyme. This includes catalytic activity which is either reduced or abolished relative to the wild type PTP. For example, the invariant aspartate residue can be changed or mutated to an alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, lysine, arginine or histidine.

10 The novel PTPs described herein, in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ), can also comprise other  
15 mutations, particularly those which assist in stabilizing the PTP/substrate complex. For example, a mutation of the [serine/threonine] residue in the signature motif to an alanine residue changes the rate-determining step of the dephosphorylation reaction from the formation of the  
20 transition state to the break down of the transition state, thereby stabilizing the PTP/substrate complex. Such mutations may be valuably combined with the replacement of the invariant aspartate residue, particularly assisting in stabilizing the complex and facilitating the observation and  
25 isolation of the complex.

PTPs suitable for use in the invention include any PTP which has an invariant aspartate residue in a corresponding position. As defined herein, a phosphatase is a member of the PTP family if it contains the signature motif  
30 [I/V]HCXAGXXR[S/T]G. Dual specificity PTPs, i.e., PTPs which dephosphorylate both phosphorylated tyrosine and phosphorylated serine or threonine, are also suitable for use in the invention. Appropriate PTPs include, but are not limited to, PTP1B, PTP-PEST, PTP $\gamma$ , MKP-1, DEP-1, PTP $\mu$ ,  
45 PTPX1, PTPX10 and PTPH1.

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In one embodiment, the invention relates to the phosphatase PTP1B in which the aspartate residue at position 181 is replaced with alanine (D181A). In another embodiment the invention relates to the phosphatase PTP-PEST in which  
5 the invariant aspartate residue at position 199 is replaced with an alanine (D199A). Another embodiment of the invention relates to a PTP-PEST phosphatase in which the cysteine residue at position 231 is replaced with a serine (C231S).

10 The invention also relates to a method of identifying a tyrosine phosphorylated protein which is a substrate of a particular protein tyrosine phosphatase. According to one embodiment of the present invention, a tyrosine phosphorylated protein of interest is combined with at least  
15 one PTP in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ) (e.g., an alanine residue), and the presence or  
20 absence of a complex between the protein and the PTP is determined. Presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the PTP. The PTP DA mutant (substrate trapping mutant) binds to or complexes with its substrate but does  
25 not dephosphorylate it (or does so very slowly), thereby allowing the complex to be isolated and identified.

The phosphorylated protein/PTP complex may be isolated by conventional isolation techniques as described in U.S. Patent No. 5,352,660 to Pawson, including salting out,  
30 chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. Furthermore, to facilitate the determination of the presence of the protein/PTP complex, antibodies against the PTP or  
35 the phosphorylated protein can be used, as well as labelled

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PTPs and/or labelled phosphorylated substrates. The FTP or phosphorylated protein can be labelled with various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include, but are not limited to, horseradish peroxidase, biotin, alkaline phosphatase,  $\beta$ -galactosidase and acetylcholinesterase. Examples of suitable fluorescent materials include, but are not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin. Appropriate luminescent materials include luminol, and suitable radioactive material include radioactive phosphorous  $^{32}\text{P}$ , iodine  $^{125}\text{I}$ ,  $^{131}\text{I}$  or tritium.

Alternatively, the invention pertains to a method of identifying a tyrosine phosphorylated protein which is a substrate of a PTP, comprising combining a PTP of interest in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ) (e.g., an alanine residue), with at least one tyrosine phosphorylated protein, thereby producing a combination; and determining the presence or absence of a complex in the combination, wherein presence of a complex in the combination between a tyrosine phosphorylated protein and the PTP indicates that the tyrosine phosphorylated protein is a substrate of the PTP.

The substrate trapping PTPs of the present invention can also be used in place of wild type PTPs to screen phosphotyrosyl peptide libraries for peptides which bind to the PTP as described in Songyang *et al.* (*Nature* 373:536-539 (1995); *Cell* 72:767-778 (1993)). Peptides identified from such peptide libraries can then be assessed to determine whether tyrosine phosphorylated proteins containing these peptides exist in nature.

Any tyrosine phosphorylated protein is suitable as a potential substrate in the present invention. Tyrosine phosphorylated proteins are well known in the art. Specific examples of appropriate substrates include, without  
5 limitation, p130<sup>cas</sup>, the EGF receptor, p210 bcr:abl, MAP kinase and the insulin receptor. Of particular interest are tyrosine phosphorylated proteins which have been implicated in a mammalian disease or disorder.

The invention also pertains to a method of reducing  
10 the activity of a tyrosine phosphorylated protein, comprising administering to a mammal a PTP in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than  
15 1 per minute (less than 1 min<sup>-1</sup>) (e.g., an alanine residue) and which forms a complex with the tyrosine phosphorylated protein. The PTP DA mutant binds to the phosphorylated protein without dephosphorylating it (or causing dephosphorylation at a greatly reduced rate), thereby  
20 inhibiting the activity of the protein and reducing its downstream effects. As used herein, "reducing" includes both reduction and complete abolishment, e.g., of one or more activities or functions of the phosphorylated protein.

For example, the invention relates to a method of  
25 reducing the transforming effects of oncogenes associated with p130<sup>cas</sup>, a substrate of PTP-PEST, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an alanine residue. Wild type PTP-PEST binds and  
30 dephosphorylates p130<sup>cas</sup>, thereby negatively regulating its downstream effects. DA mutants of PTP-PEST bind but do not dephosphorylate p130<sup>cas</sup> (or do so at a greatly reduced rate); the substrate is thus tied up in the complex with the substrate trapping form of PTP-PEST and cannot exert its  
35 downstream effects. Similarly, the invention relates to a



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method of reducing the formation of signalling complexes associated with  $p130^{cas}$ , particularly those signalling complexes which induce mitogenic pathways, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an alanine residue. The PTP binds to and/or dephosphorylates  $p130^{cas}$ , thereby negatively regulating the downstream effects of  $p130^{cas}$  and reducing the formation of signalling complexes associated with  $p130^{cas}$ .

The substrate trapping mutant PTPs of the present invention can be used in virtually any application in place of, or in addition to, a corresponding wild type PTP. The advantages of such a utility lie in the ability of the mutant PTP to mimic the function of the wild type enzyme, e.g., to decrease the activity of its tyrosine phosphorylated substrate, without inducing the harmful cytotoxic effects commonly observed with administration or overexpression of the wild type PTP. Thus, the invention also pertains to a method of reducing the cytotoxic effects associated with administration or overexpression of wild type PTPs. For example, CS mutants of MKP-1 have been shown to have the same functional effect as wild type MKP-1 without induction of potentially harmful side effects. Thus, PTPs described herein, in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ) (e.g., an alanine residue), can be used in many applications in place of the corresponding wild type enzyme. As used herein, a "corresponding" enzyme is one which is the same as the mutant PTP (e.g., PTP-PEST and PTP-PEST D199A) or one which is different from the mutant PTP but recognizes the same substrate as the mutant PTP.

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The mutant PTPs described herein can also be used therapeutically to reduce the activity of a tyrosine phosphorylated protein, such as by a gene therapy method in which the mutant PTP described herein, or a functional  
5 portion thereof which retains the ability to bind to its tyrosine phosphorylated substrate, is introduced into a subject and in whom the mutant PTP is expressed. The mutant PTP replaces, either partially or totally, the wild type enzyme which is normally produced or competes with the wild  
10 type PTP for binding to the substrate. For example, a specific tyrosine phosphorylated protein can be identified which is implicated in a particular disease or disorder (such as a protein tyrosine kinase). At least one PTP which acts to dephosphorylate the selected tyrosine phosphorylated  
15 protein of the present invention can be identified by the methods described herein. The wild type or mutant form of the PTP can be administered to a subject in need of treatment in order to tie up or bind the tyrosine phosphorylated substrate, thereby inhibiting or reducing the  
20 function of the phosphorylated protein. In a preferred embodiment, the mutant PTP is administered in place of the wild type enzyme in order to reduce the cytotoxic effects associated with overexpression of the wild type enzyme. Procedures for gene therapy are known in the art (see U.S.  
25 Patent No. 5,399,346 to Anderson et al.) and can be modified by methods known in the art to appropriately express the specific mutant and wild type PTPs of the present invention.

The present invention also relates to assays for identifying agents which alter, e.g., enhance or inhibit,  
30 the interaction between a PTP and its phosphorylated substrate. Agents identified by these assays can be agonists (e.g., agents which enhance or increase the activity of the PTP) or antagonists (e.g., agents which inhibit or decrease the activity of the PTP) of PTP  
35 activity. The agent may be an endogenous physiological

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substance or may be a natural or synthetic drug, including small organic molecules.

For example, the tyrosine phosphorylated substrate of a PTP can be identified by the methods described herein. An enzymatic activity assay utilizing the wild type PTP can be carried out in the presence of an agent to be tested, and the resulting amount of enzyme activity can be compared with the amount of enzyme activity in the absence of the agent to be tested. Enzymatic activity assays are known in the art; for example, assays of PTP activity using a tyrosine phosphorylated <sup>32</sup>P-labelled substrate are described in Flint *et al.* (*EMBO J.* 12:1937-1946 (1993)). A decrease in the enzymatic activity in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate. Conversely, an increase in the enzymatic activity in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Alternatively, a competitive binding assay can be carried out utilizing the mutant PTP in the presence of an agent to be tested, and the resulting extent of binding of the mutant PTP to its substrate can be compared with the extent of binding in the absence of the agent to be tested. Competitive binding assays are known in the art; for example, U.S. Patent No. 5,352,660 to Pawson describes methods suitable for use in this invention. A decrease in the extent of binding in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate. Conversely, an increase in the extent of binding in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

According to the present invention, tyrosine phosphorylated peptides identified with mutant PTPs from peptide libraries by the methods of Songyang *et al.* (*Nature*

373:536-539 (1995); Cell 72:767-778 (1993)) can be used herein in place of the complete tyrosine phosphorylated protein in competitive binding assays.

The present invention also pertains to pharmaceutical compositions comprising a PTP in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute (less than  $1 \text{ min}^{-1}$ ) (e.g., an alanine residue). For instance, the PTP of the present invention can be formulated with a physiologically acceptable medium to prepare a pharmaceutical composition. The particular physiological medium may include, but is not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists, and will depend on the ultimate pharmaceutical formulation desired. Methods of introduction of exogenous PTPs at the site of treatment include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral and intranasal. Other suitable methods of introduction can also include rechargeable or biodegradable devices and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other agents.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated by reference in their entirety.

EXAMPLESMaterials and Methods

The following is a description of the materials and methods used in the work described herein.

5 Generation, Expression and Purification of Mutant PTP  
10 Proteins

Point mutations within the catalytic domains of PTP-  
PEST (D199A, C231S) and PTP1B (D181A, C215S) were introduced  
by site-directed mutagenesis using the Muta-Gene<sup>TM</sup> in vitro  
10 mutagenesis kit (Bio-Rad, Richmond, CA). Regions containing  
the required point mutation were then exchanged with the  
wild type sequences within appropriate expression vectors,  
and the replaced mutant regions were sequenced in their  
entirety to verify the absence of additional mutations.

15 Full length PTP-PEST proteins (wild type and mutant  
proteins, containing either Asp199 to Ala or Cys231 to Ser  
mutations) and the wild type PTP-PEST catalytic domain  
(amino acids 1-305) were expressed in Sf9 cells using  
recombinant baculovirus (BaculoGold<sup>TM</sup>, Pharmingen, San  
20 Diego, CA), and purified as described in Garton and Tonks  
(EMBO J. 13:3763-3771 (1994)). Truncated forms of wild type  
and mutant PTP-PEST proteins, comprising amino acid residues  
1-305 of PTP-PEST were also expressed in E. coli as GST  
fusion proteins following subcloning of PTP-PEST DNA in-  
25 frame downstream of GST in pGEX vectors (Pharmacia Biotech  
Inc., Uppsala, Sweden). Twenty-five ml of E. coli  
transformed with the appropriate vector were grown to log  
phase (OD<sub>600</sub> approximately 0.5). Fusion protein expression  
was then induced by addition of 0.2 mM isopropyl-1-thio-β-D  
30 galactopyranoside, and the cells were grown for 2-4 hours at  
30°C. Cells were harvested by centrifugation, incubated  
with 50 mg/ml lysozyme in 3 ml buffer containing 50 mM Tris-  
HCl, pH 7.4, 5mM EDTA, 1 mM PMSF, 1 mM benzamidine, 5 mg/ml  
leupeptin, 5 mg/ml aprotinin, 0.1 % Triton X-100 and 150 mM

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NaCl, then lysed by sonication (3 x 10s). Following removal of insoluble material by centrifugation (20 minutes at 300,000 x g), fusion proteins were isolated by incubation for 30 min at 4°C with 100 ml glutathione-Sepharose beads (Pharmacia Biotech Inc., Uppsala, Sweden), and the beads were then collected by centrifugation and washed three times with buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 % glycerol, 1 % Triton X-100 and 100 mM NaCl). This procedure yielded essentially homogeneous fusion protein at a concentration of 1 mg protein/ml glutathione-Sepharose beads. PTP1B proteins (wild type and mutant forms) comprising amino acids 1-321 were expressed in *E.coli* and purified to homogeneity as described in Barford *et al.* (*J. Mol. Biol.* 239:726-730 (1994)).

#### Cell Culture, Transfection, Preparation of Lysates and Fractionation

HeLa and COS cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 5% fetal bovine serum (FBS); Wi38, C2C12 and MvLu cells were grown in DMEM containing 10% FBS; 293 cells were grown in DMEM containing 10% calf serum; MCF10A cells were grown in 50% DMEM, 50% Ham's F-12 containing 5% horse serum, 20 ng/ml epidermal growth factor, 10 mg/ml insulin, 0.5 mg/ml hydrocortisone and 0.25 mg/ml fungizone. All media also contained penicillin and streptomycin at 100 U/ml and 100 mg/ml, respectively, and all cells were grown at 37°C. Calcium phosphate-mediated transfection was used to introduce cDNA encoding wild type and mutant PTP-PEST proteins into COS cells. These were encoded by PTP-PEST cDNA subcloned into the plasmid PMT2, from which expression was driven by an adenovirus major late promoter; 20 mg DNA was used for transfection of each 10 cm plate of cells. The level of expression of PTP-PEST constructs was similar in all cases.

Prior to cell lysis, 70-90% confluent cultures of cells were treated for 30 minutes with 0.1 mM pervanadate (20 ml of a fresh solution containing 50 mM sodium metavanadate ( $\text{NaVO}_3$ ) and 50 mM  $\text{H}_2\text{O}_2$  were added to 10 ml medium). Treatment of cells with  $\text{H}_2\text{O}_2$  and vanadate leads to a synergistic increase in phosphotyrosine levels, presumably due to inhibition of intracellular PTPs by vanadate. The synergism between  $\text{H}_2\text{O}_2$  and vanadate has previously been suggested to result from improved accumulation of the resultant oxidized vanadate (pervanadate) within the cells when compared to vanadate itself (Heffetz *et al.*, *J. Biol. Chem.* 265:2896-2902 (1990)). It is important to note that during the preparation of cell lysates, dilution occurs such that the inhibitory effect of vanadate on PTP action is lost. Pervanadate treatment resulted in the appearance of at least 50 prominent phosphotyrosine protein bands in all cell types, whereas untreated cells contained virtually undetectable levels of phosphotyrosine (data not shown).

Cells were lysed in Buffer A containing 5 mM iodoacetic acid, which was included in order to inhibit irreversibly cellular PTPs. Following incubation at 4°C for 30 minutes, 10 mM DTT was added to inactivate any unreacted iodoacetic acid. Insoluble material was then removed by centrifugation for 20 minutes at 300,000 x g. The resultant lysates were stable with regard to their phosphotyrosine content during long term (several months) storage at -70°C and during prolonged (at least 20 hours) incubation at 4°C, in the absence of exogenous added PTPs.

Pervanadate-treated HeLa cell lysate was fractionated by anion exchange chromatography using a Mono Q FPLC column (Pharmacia). The sample (50 mg total protein at 3 mg/ml in buffer A) was diluted in three volumes of buffer B (20 mM tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 0.1% Triton X-100) prior to

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loading. Proteins were eluted at a flow rate of 1 ml/min with a linear gradient of 0-0.5 M NaCl in buffer B over 20 fractions (1 ml fraction volume), followed by a second gradient of 0.5-1.0 M NaCl in buffer B over 5 fractions.

- 5 Phosphotyrosine-containing proteins were detected within fractions 7-21 according to anti phosphotyrosine immunoblotting. The same procedures were followed for PTP1B, with the exception that the cells were not treated with pervanadate.

10 Dephosphorylation Reactions

- Lysates of pervanadate-treated HeLa cells (1-2 mg protein/ml) containing tyrosine phosphorylated proteins were incubated on ice in the absence or presence of purified active PTPs at a concentration of 2 nM. Dephosphorylation  
15 was terminated by the removal of aliquots (30 mg protein) into SDS-PAGE sample buffer, and the extent of dephosphorylation was determined by immunoblotting using the monoclonal antibody G104. Assays of PTP activity using tyrosine phosphorylated <sup>32</sup>P-labelled RCM-lysozyme as  
20 substrate were performed as described in Flint et al. (EMBO J. 12:1937-1946 (1993)).

Antibodies and Immunoblotting

- The PTP-PEST monoclonal antibody AG25 was raised against baculovirus-expressed purified full-length PTP-PEST.  
25 The anti-phosphotyrosine monoclonal antibody G104 was generated using as antigen phosphotyrosine, alanine and glycine, in a 1:1:1 ratio, polymerized in the presence of keyhole limpet hemocyanin with 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide, a method originally  
30 described in Kamps and Sefton (Oncogene 2:305-315 (1988)). p130<sup>cas</sup> monoclonal antibody was from Transduction Laboratories (Lexington, Ky). Monoclonal antibody FG6 against PTP1B was provided by Dr David Hill (Calbiochem



Oncogene Research Products, Cambridge, MA). Visualization of proteins by immunoblotting was achieved by enhanced chemiluminescence (ECL) using HRP-conjugated secondary antibodies (Amersham Life Science Inc., Arlington Heights, IL) and the SuperSignal<sup>TM</sup> CL-HRP substrate system (Pierce, Rockford, IL).

#### Immunoprecipitation and Substrate Trapping

Immunoprecipitation of PTP-PEST from transfected COS cells was performed following covalent coupling of monoclonal antibody AG25 to protein A-Sepharose beads (Pharmacia Biotech Inc., Uppsala, Sweden) using the chemical cross-linking agent dimethyl pimelimidate (Schneider *et al.*, *J. Biol. Chem.* 257:10766-10769 (1982)). Antibody was first bound to protein A Sepharose at a concentration of 1 mg/ml bead volume, and unbound material was then removed by three washes with 0.2 M sodium borate, pH 9. Covalent coupling was achieved by incubation at room temperature for 30 minutes in the presence of 20 mM dimethyl pimelimidate in 0.2 M sodium borate, pH 9. The beads were then incubated for 1 hour with an excess of 0.2 M ethanolamine, pH 8, to block any unreacted cross-linker, and washed three times with PBS prior to storage at 4°C. Ten ml of AG25 beads were used to precipitate transfected PTP-PEST from lysates containing approximately 0.375 mg protein.

Substrate trapping was performed using various PTP affinity matrices. The full-length PTP-PEST matrix utilized covalent coupled AG25-protein A-Sepharose beads to which purified baculovirus-expressed PTP-PEST protein was bound. Aliquots (10ml) of AG25 beads were incubated for 2 hours at 4°C in 100 ml buffer A in the presence of 5 mg of purified PTP-PEST (wild type or mutant forms); unbound PTP-PEST was then removed by washing three times with 1 ml buffer A. The resultant PTP-PEST-AG25-protein A-Sepharose beads contained approximately 2 mg of PTP-PEST per 10 ml aliquot. Substrate

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trapping was also carried out with glutathione-Sepharose beads bound to bacterially-expressed GST fusion proteins containing the catalytic domain of PTP-PEST.

PTP1B was also used in substrate trapping experiments.

- 5 In this case, the monoclonal antibody FG6 was precoupled to protein A-Sepharose in the absence of cross-linker (2 mg antibody/10 ml beads), then purified PTP1B proteins were added in excess and incubated at 4°C for 2 hours. Following removal of unbound PTP1B, 10 ml beads contained
- 10 approximately 2 mg PTP1B.

- Pervanadate-treated cell lysates, or column fractions, were used as a source of phosphotyrosine-containing proteins for substrate trapping experiments. In general, lysates containing 0.25-0.5 mg protein in 0.5 ml buffer A (including
- 15 5 mM iodoacetic acid, 10 mM DTT) were incubated at 4°C for 2 hours in the presence of 10 ml of affinity matrix containing approximately 2 mg of the appropriate PTP protein. Unbound proteins were then removed from the samples by washing three times with 1 ml buffer A, and bound material was collected
- 20 by addition of 50 ml SDS-PAGE sample buffer followed by heating at 95°C for 5 minutes; proteins bound to the beads were then analyzed by SDS-PAGE followed by immunoblotting.

### Results

- The following details the results of the work
- 25 described herein carried out as described above.

#### PTP1B and p210 bcr:abl

- Chronic myelogenous leukemia (CML) is a clonal disorder of the haematopoietic stem cell that is characterized by the Philadelphia chromosome, in which the
- 30 c-Abl proto-oncogene on chromosome 9, encoding a PTK, becomes linked to the bcr gene on chromosome 22. This results in the generation of a bcr:abl fusion protein, p210 bcr:abl, in which the PTK activity is enhanced relative to

that of c-Abl. Current data indicates that this cytogenetic abnormality is the primary and sole causative event in CML. Expression of p210 bcr:abl produces abnormal patterns of tyrosine phosphorylation that result in the aberrant  
5 maturation of the haematopoietic stem cell that is characteristic of CML.

Expression of PTP1B mRNA and protein is enhanced as a consequence of p210 bcr:abl expression in Rat1, Mo7 and BaF3 cells. Changes in PTP1B activity, which were commensurate  
10 with the change in enzyme protein, were also observed. These changes are specific for PTP1B and are not seen in closely related homologue (65% identity) TC-PTP or in other tested PTPs, including SHP-1, SHP-2 and PTP-PEST. The increase in expression of PTP1B was also observed in Ph+ B-  
15 lymphoid cells derived from a CML patient relative to Ph-cells from the same patient.

The changes in PTP1B levels were induced specifically by p210 bcr:abl and were not seen in cells expressing other PTKs including v-abl, v-src or other oncoproteins such as  
20 myc. The PTK activity of p210 bcr:abl was essential for the increase in expression of PTP1B, since expression of an inactive lysine to arginine mutant form of p210 bcr:abl in Rat1 cells did not alter PTP1B levels. The increase in PTP1B levels is a rapid response to induction of p210  
25 bcr:abl. When BaF3 cells expressing a temperature-sensitive mutant form of p210 bcr:abl were shifted to the permissive temperature for the PTK, PTP1B levels were observed to increase within 12-24 hours coincident with the appearance of the active form of the PTK. These data indicate that the  
30 alteration PTP1B levels is a relatively rapid response to the appearance of p210 bcr:abl, rather than a long-term adaptive response of the cells.

In transient cotransfection experiments in COS cells, PTP1B dephosphorylates p210 bcr:abl but not v-abl. When the  
35 PTP1B D181A mutant was expressed as a GST fusion protein,

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purified and incubated with lysates of Mo7-p210 cells (which overexpress p210 bcr:abl), a complex of the mutant PTP and p210 bcr:abl was isolated. In contrast, tyrosine phosphorylated c-abl, which was also present in the lysates, did not bind to the mutant PTP. The interaction between PTP1B D181A and p210 bcr:abl was blocked by vanadate, suggesting that the interaction involved the active site of the PTP.

Following transient coexpression in COS cells, PTP1B D181A formed a complex with p210 bcr:abl. Preliminary data indicate that the Y177F mutant form of p210 bcr:abl did not interact with PTP1B D181A, suggesting that this tyrosine residue is a component of the binding site in the PTK. This tyrosine residue in p210 bcr:abl is phosphorylated in vivo and has been demonstrated to serve as a docking site for GRB2. Direct interaction of the pTyr in p210 bcr:abl and the SH2 domain of GRB2 is essential for the transforming activity of the PTK. Interaction of PTP1B D181A with p210 bcr:abl interferes with the association of the PTK with GRB2. Taken together, these data suggest that p210 bcr:abl is a physiological substrate of PTP1B and that PTP1B may function as an antagonist of the oncoprotein PTK in vivo. The Vmax, Km and Kcat of 37 kDa PTP1B mutants toward RCML are shown in Figure 2.

#### 25 PTP1B and the EGF Receptor

Expression of PTP1B D181A in COS cells leads to enhanced phosphorylation of tyrosyl residues in a 180 kDa protein and in proteins of 120 and 70 kDa. When a GST-PTP1B D181A fusion protein is expressed in COS cells and precipitated on Glutathione-Sepharose, the 180 kDa, and smaller quantities of p120 and p70, were coprecipitated. The p180 protein was identified as the epidermal growth factor (EGF) receptor by immunoblotting. The identity of

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the p120 and p70 proteins is unclear; however, the latter is not src, p62 or paxillin.

Expression of PTP1B D181A in COS cells induces tyrosine phosphorylation of the EGF receptor in the absence  
5 of its ligand, EGF, indicating that the mutant PTP is exerting its effects in the intact cell and not post-lysis. The equivalent D199A PTP-PEST mutant does not interact with the EGF receptor, indicating the specificity of this substrate interaction.

10 Autophosphorylation of the EGF receptor is required for the interaction with PTP1B D181A. Mutants of the receptor that are either kinase-dead or in which the autophosphorylation sites have been deleted do not interact with PTP1B D181A. In v-src-expressing cells, a plethora of  
15 tyrosine phosphorylated proteins were observed, but phosphorylation of the EGF receptor was not detected. Under these conditions, PTP1B D181A bound predominantly to a 70 kDa tyrosine phosphorylated protein.

As a result of this work, it appears that PTP1B can  
20 modulate EGF-induced signalling pathways, perhaps including the pathways of many diseases, including breast cancer.

#### Preferential Dephosphorylation of a 130 kDa Phosphotyrosine-Containing Protein by PTP-PEST

In order to investigate the substrate specificity of  
25 PTP-PEST in vitro, aliquots of pervanadate-treated HeLa cell lysates were incubated on ice, yielding 50-100 distinct phosphotyrosine-containing proteins as judged by immunoblotting of the cell lysate using the monoclonal anti-phosphotyrosine antibody G104. Purified full length PTP  
30 PEST (expressed in Sf9 cells using recombinant baculovirus), PTP-PEST catalytic domain, or PTP1B catalytic domain (37 kDa form) was then added to the lysate, and aliquots were removed at various time points for analysis by SDS-PAGE followed by anti-phosphotyrosine immunoblotting.

Surprisingly, a prominent 130 kDa phosphotyrosine band (p130) was selectively dephosphorylated by PTP-PEST within 10 minutes, whereas the intensity of all the other bands was essentially unchanged even after 60 minutes of incubation with PTP-PEST. Long incubations with higher concentrations of PTP-PEST (greater than 100-fold) resulted in the complete removal of all phosphotyrosine bands from the lysate. However, under all conditions tested, p130 was found to be dephosphorylated more rapidly than all other bands present.

10 The selective dephosphorylation of p130 by PTP-PEST was also observed using a truncated form of the phosphatase (amino acid residues 1-305) which essentially contains only the catalytic domain of the enzyme. This result suggests that the striking substrate preference displayed by PTP-PEST  
15 in this analysis is an inherent property of the phosphatase catalytic domain, whereas the C-terminal 500 amino acid residues have little discernible effect on the substrate specificity of the enzyme.

The specificity of the interaction between PTP-PEST  
20 and p130 was addressed using the catalytic domain of PTP1B (amino acid residues 1-321) in dephosphorylation reactions. When added at a similar molar concentration to that used for PTP-PEST, PTP1B was found to dephosphorylate fully and rapidly (within 15 minutes) most of the phosphotyrosine-  
25 containing proteins present in the pervanadate-treated HeLa lysate. In addition, the time course of dephosphorylation of p130 was not significantly more rapid than that of the other phosphotyrosine bands dephosphorylated by PTP1B. It should be noted, however, that these in vitro  
30 dephosphorylation results are not truly illustrative of the substrate specificity of PTP1B in vivo for several reasons. First, only the isolated catalytic subunit was used in this particular experiment. Furthermore, in vivo substrate  
35 specificity may be quite different due to the intracellular distribution of both the PTP and potential substrates. That

is, in vitro dephosphorylation experiemnts may utilize substrates which the PTP is capable of dephosphorylating but which it would not have access to in vivo. The phenomenon of differing substrate specificity depending upon different physiologic contexts is illustrated by a comparison of this data with the in vivo PTP1B work described above, wherein PTP1B showed specificity for only three proteins.

Identification of Phosphotyrosine-Containing p130 Protein as p130<sup>cas</sup> by Substrate Trapping

10 Pervanadate-treated HeLa cell lysate was fractionated by anion exchange chromatography and aliquots of the fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-p130<sup>cas</sup> antibodies. Aliquots of all samples analyzed were then  
15 incubated with an affinity matrix containing a substrate trapping PTP-PEST mutant, comprising full length PTP-PEST in which Asp199 is changed to alanine (D199A), bound to covalently coupled protein A-Sepharose/antibody (AG25) heads. Proteins associated with PTP-PEST were then analyzed  
20 by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-p130<sup>cas</sup> antibodies.

Anti-phosphotyrosine immunoblotting of the column fractions showed that the p130 phosphotyrosine band eluted as a single peak in fractions 11-14 (approx. 0.3 M NaCl).  
25 In view of the abundance of tyrosine phosphorylated p130 in HeLa lysates, it appeared likely that p130 represents a previously identified phosphotyrosine-containing 130 kDa protein. Several potential candidates were identified in the literature, including the focal adhesion kinase  
30 p125<sup>FAK</sup>, ras-GAP, qpl30 and p130<sup>cas</sup>. Of these candidates, p130<sup>cas</sup> has been identified as a particularly prominent phosphotyrosine band in a wide variety of systems, including v-crk (Mayer and Hanafusa, Proc. Natl. Acad. Sci. USA 87: 2638-2642 (1990); Mayer et al., Nature 332:272-275 (1988))

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and src (Kanner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3328-3332 (1990); Reynolds *et al.*, *Mol. Cell. Biol.* 9: 3951-3958 (1989)) transformed fibroblasts, integrin mediated cell adhesion (Nojima *et al.*, *J. Biol. Chem.* 270:15398-15402 (1995); Petch *et al.*, *J. Cell Science* 108:1371-1379 (1995); Vuori and Ruoslahti, *J. Biol. Chem.* 270:22259-22262 (1995)) and PDGF stimulated 3T3 cells (Rankin and Rozengurt, *J. Biol. Chem.* 269:704-710 (1994)).

Therefore, the possibility that the p130  
10 phosphotyrosine band corresponds to p130<sup>cas</sup> was tested by immunoblotting the Mono Q fractions using an antibody to p130<sup>cas</sup>. The 130 kDa band corresponding to p130<sup>cas</sup> eluted in the same fractions as the p130 tyrosine phosphorylated band, and displayed a similar apparent molecular weight,  
15 suggesting that they might represent the same protein. Furthermore, p130<sup>cas</sup> immunoprecipitated from these fractions was found to be phosphorylated on tyrosyl residues.

A mutant form of PTP-PEST (D199A) was generated by  
20 site-directed mutagenesis, and the mutant enzyme was purified following expression using recombinant baculovirus. When assayed using tyrosine phosphorylated RCM-Lysozyme as substrate, the purified mutant enzyme exhibited a specific activity which was approximately 10,000 fold lower than  
25 that of the wild type enzyme (Garton and Tonks, unpublished data). This purified protein was bound to an affinity matrix comprised of an anti-PTP-PEST monoclonal antibody (AG25) covalently coupled to Protein A-Sepharose beads, then incubated with each of the Mono Q fractions. After 45  
30 minutes of incubation, proteins associating with the mutant PTP-PEST were collected by centrifugation, the beads were washed, and SDS-PAGE sample buffer was added. Associated proteins were then analyzed by immunoblotting using the monoclonal anti-phosphotyrosine antibody G104.



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The mutant PTP-PEST protein was found to associate with a single phosphotyrosine-containing protein, the molecular weight (130 kDa) and Mono Q elution position (fractions 11-14) of which coincided with those of p130<sup>cas</sup>.  
5 Immunoblotting of the PTP-PEST-associated proteins using the p130<sup>cas</sup> antibody demonstrated that the 130 kDa tyrosine phosphorylated protein trapped by the mutant PTP-PEST is indeed p130<sup>cas</sup>. These data further support the hypothesis that p130<sup>cas</sup> is a potential physiologically relevant  
10 substrate for PTP-PEST.

Determination of Structural Features of PTP-PEST Involved in Specific Interaction with Tyrosine Phosphorylated p130<sup>cas</sup>

The interaction between p130<sup>cas</sup> and PTP-PEST was investigated further in substrate trapping experiments using  
15 various purified mutant forms of PTP-PEST to precipitate proteins from pervanadate-treated HeLa lysates. Several affinity matrices were incubated with pervanadate-treated HeLa cell lysate, and proteins associated with the beads were analyzed by SDS-PAGE followed by immunoblotting with  
20 anti-phosphotyrosine or anti-p130<sup>cas</sup> antibodies.

The wild type full-length phosphatase was found to be incapable of stable association with tyrosine phosphorylated p130<sup>cas</sup>, whereas both the PTP-PEST (D199A) mutant protein and a mutant lacking the active site cysteine residue  
25 (C231S) specifically precipitated p130<sup>cas</sup> from the lysate. The inability of the wild type phosphatase to precipitate tyrosine phosphorylated p130<sup>cas</sup> presumably reflects the transient nature of the normal interaction between PTP-PEST and tyrosine phosphorylated p130<sup>cas</sup>, which is likely to be  
30 concluded as soon as p130<sup>cas</sup> is dephosphorylated by PTP-PEST.

Since the C-terminal 500 amino acids of PTP-PEST contain several proline-rich regions which resemble src homology-3 (SH3) domain binding sequences, it appeared

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plausible that the specificity of the interaction between PTP-PEST and p130<sup>cas</sup> might depend to some extent on association of these segments with the SH3 domain of p130<sup>cas</sup>. The possible contribution of the C-terminal  
5 segment of PTP-PEST in the observed specific interaction of PTP-PEST with p130<sup>cas</sup> was therefore addressed in further substrate trapping experiments using GST fusion proteins containing the catalytic domain of PTP-PEST alone, in both wild type and mutant (D199A) forms. The mutant catalytic  
10 domain of PTP-PEST fused to GST was found to precipitate the p130<sup>cas</sup> phosphotyrosine band specifically, whereas both the wild type fusion protein and GST alone failed to precipitate p130<sup>cas</sup>. The specific interaction between PTP-PEST and p130<sup>cas</sup> observed in these experiments therefore appears to  
15 be an intrinsic property of the catalytic domain of PTP-PEST, emulating the observed preference of the active PTP-PEST catalytic domain for dephosphorylation of p130<sup>cas</sup> in vitro.

Specificity of Interaction Between Mutant PTP-PEST and  
20 Tyrosine Phosphorylated p130<sup>cas</sup>

In view of the relative abundance of tyrosine phosphorylated p130<sup>cas</sup> in the pervanadate-treated HeLa cell lysate, the possibility that the observed selective binding of PTP-PEST inactive mutant proteins to p130<sup>cas</sup> was  
25 substrate-directed (reflecting the abundance of this potential substrate relative to the other phosphotyrosine-containing proteins present in the lysate) rather than enzyme-directed (reflecting a genuine substrate preference of PTP-PEST) was considered; this possibility was addressed  
30 in two ways. First, inactive mutant forms of the catalytic domain of PTP1B were used to trap potential substrates for this enzyme from the pervanadate-treated HeLa lysates. Again it was found that the wild type phosphatase was incapable of stable interaction with any phosphotyrosine-

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containing protein, whereas mutant variants of the PTP1B phosphatase domain (comprising Cys or Asp mutations analogous to those described above for PTP-PEST) associated with many tyrosine phosphorylated proteins. This was especially apparent for the aspartic acid mutant of PTP1B (D181A), which appeared to precipitate essentially all phosphotyrosine-containing proteins from the lysate with similar efficacy. These data emphasize the specific nature of the interaction between PTP-PEST and p130<sup>cas</sup>, which appears to be a property peculiar to the PTP-PEST catalytic domain, rather than a feature shared by all PTP catalytic domains.

The specificity of the interaction between PTP-PEST and p130<sup>cas</sup> was addressed further following pervanadate-treatment of several different cell lines (Wi38, 293, COS, MCF10A, C2C12, MvLu), yielding a different array of tyrosine phosphorylated proteins in each case; the resultant lysates were analyzed by SDS-PAGE followed by anti-phosphotyrosine immunoblotting. Aliquots were incubated with PTP-PEST (D199A) affinity matrix or control matrix, and tyrosine phosphorylated proteins associating with PTP-PEST were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine or anti-p130<sup>cas</sup> antibodies as described above.

In each case, the D199A mutant PTP-PEST protein precipitated a single broad phosphotyrosine band with an apparent molecular weight between 120 and 150 kDa in different cell lines, whereas the affinity matrix alone failed to precipitate any phosphotyrosine-containing protein. Immunoblotting of the precipitates with a p130<sup>cas</sup> antibody revealed that the protein precipitated from all cell lysates corresponded to p130<sup>cas</sup>; the observed molecular weight variation between different cell lines presumably reflects either species differences in the molecular weight of p130<sup>cas</sup> or expression of different

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alternatively spliced forms (Sakai *et al.*, *EMBO J.* 13:3748-3756 (1994)).

The relative abundance of tyrosine phosphorylated p130<sup>cas</sup> in the PTP-PEST precipitates appeared to correlate approximately with the abundance of p130<sup>cas</sup> protein in the lysates (data not shown). Surprisingly, regardless of the abundance of tyrosine phosphorylated p130<sup>cas</sup> in the lysates, p130<sup>cas</sup> was invariably the only phosphotyrosine-containing protein in the precipitates, even in 293 cell lysates which contained very little p130<sup>cas</sup> protein but which displayed a wide variety of other abundantly tyrosine phosphorylated proteins. Similarly, when lysates of pervanadate-treated 293 cells (containing tyrosine phosphorylated p130<sup>cas</sup> in amounts which are undetectable by anti-phosphotyrosine immunoblotting of the lysate) were incubated with active PTP-PEST, no visible dephosphorylation of any phosphotyrosine band occurred (Garton and Tonks, unpublished data). These results indicate that the affinity of PTP-PEST for p130<sup>cas</sup> is substantially greater than for any other substrate present, and further emphasizes the remarkable substrate selectivity of PTP-PEST for p130<sup>cas</sup>.

#### Vanadate Inhibition of Tyrosine Phosphorylated p130<sup>cas</sup> Association with Mutant PTP-PEST

A consistent observation of this work was that, in contrast to the inactive mutant PTP-PEST, the wild type enzyme failed to associate in a stable complex with tyrosine phosphorylated p130<sup>cas</sup>, suggesting that the observed association is active site-directed. In order to investigate this possibility, mutant PTP-PEST (D199A) was incubated with the PTP inhibitor vanadate at various concentrations prior to addition of pervanadate-treated HeLa cell lysate. The extent of association of p130<sup>cas</sup> with PTP-PEST was then analyzed. PTP-PEST affinity matrix, comprising full length PTP-PEST (D199A) bound to covalently

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coupled protein A-Sepharose/antibody (AG25) beads, was incubated for 10 minutes on ice in the presence of varying concentrations of sodium orthovanadate. The samples were then incubated with aliquots of pervanadate-treated HeLa  
5 cell lysate; associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine or anti-p130<sup>cas</sup> antibodies. The activity of wild type PTP-PEST was also determined under the same conditions, using tyrosine phosphorylated <sup>32</sup>P-labelled RCM-lysozyme as substrate.

10 The association was found to be potently disrupted by vanadate, with a concentration-dependence similar to that of vanadate inhibition of wild type PTP-PEST, and complete disruption being observed at 10 mM vanadate. Since PTP inhibition by vanadate presumably results from a direct  
15 interaction of vanadate with the active site cysteine residue of the enzyme (Denu et al., Proc. Natl. Acad. Sci. USA 93:2493-2498 (1996)), this result supports the hypothesis that the stable association of mutant PTP-PEST with tyrosine phosphorylated p130<sup>cas</sup> is mediated by direct  
20 interactions between active site residues within PTP-PEST, in particular the active site cysteine residue, and phosphotyrosine moieties within p130<sup>cas</sup>.

#### Association of Endogenous p130<sup>cas</sup> with Transfected Mutant PTP-PEST in COS Cells

25 The work described above strongly suggests that p130<sup>cas</sup> represents a potential physiologically significant substrate for PTP-PEST. In order to assess whether PTP-PEST interacts with p130<sup>cas</sup> in intact cells, COS cells were transfected with plasmids encoding wild type or mutant forms  
30 of PTP-PEST (D199A or C215S). The cells were treated with pervanadate 30 minutes prior to lysis, PTP-PEST proteins were immunoprecipitated, and associated tyrosine phosphorylated proteins were analyzed by anti-phosphotyrosine immunoblotting of the resultant

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precipitates. Lysates were also incubated with covalently coupled protein A-Sepharose/anti-PTP-PEST (AG25) beads and associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody.

5 Under these conditions, the phosphotyrosine-containing band corresponding to p130<sup>cas</sup> was again unique in its ability to associate with the C231S PTP-PEST protein, indicating that p130<sup>cas</sup> can be specifically selected by PTP-PEST as a substrate in an intracellular context in the  
10 presence of a large number of alternative possible substrates. Neither the wild type nor the D199A form of PTP-PEST was capable of a stable interaction with tyrosine phosphorylated p130<sup>cas</sup> in pervanadate-treated COS cells.

The binding of both wild type and D199A PTP-PEST to  
15 tyrosine phosphorylated p130<sup>cas</sup> under these conditions is most likely prohibited by the presence of pervanadate bound to the active site cysteine residue of PTP-PEST (Denu *et al.*, Proc. Natl. Acad. Sci. USA 93:2493-2498 (1996)), which effectively excludes the binding of phosphotyrosine residues  
20 of p130<sup>cas</sup>. The ability of the C231S mutant PTP-PEST to associate in a stable complex with p130<sup>cas</sup> in the presence of pervanadate suggests that this mutant protein is largely unaffected by pervanadate, indicating that the normal mode of inhibition of PTPs by vanadate ions depends critically on  
25 direct interactions between vanadate and the thiolate anion of the PTP active-site cysteine residue. These observations therefore lend further support to the existence of an exclusive interaction between PTP-PEST and p130<sup>cas</sup> which appears to be entirely active site-directed, and therefore  
30 reflects the genuine, inherent, highly restricted substrate preference of PTP-PEST for p130<sup>cas</sup>.

Results described herein implicate p130<sup>cas</sup> as a physiologically relevant substrate for PTP-PEST. Furthermore, the observed stringency and exclusivity of the  
35 interaction between PTP-PEST and p130<sup>cas</sup> in a wide variety

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of cell lines suggest that p130<sup>cas</sup> may be a unique high affinity substrate for PTP-FEST, although the possibility that other significant PTP-FEST substrates may exist cannot be excluded at present. In particular, it is unclear whether pervanadate-treated cells display a complete spectrum of all possible tyrosine phosphorylated proteins; in fact this appears unlikely since pervanadate treatment presumably results only in an increase in tyrosine phosphorylation of proteins which are to some extent constitutively phosphorylated, but which are normally rapidly dephosphorylated, within the cell. Potential substrates lacking from pervanadate-treated cells therefore presumably include substrates of protein tyrosine kinases (PTKs) which are normally present in an inactive state, such as ligand stimulated receptor PTKs, and the recently described calcium regulated kinase PYK2 (Lev et al., Nature 376: 737-745 (1995)). Regardless of these considerations, the ability of PTP-FEST to select p130<sup>cas</sup> exclusively as a substrate from lysates of several different cell lines, containing a combined total of at least one hundred different potential substrates (many of which presumably contain multiple sites of phosphorylation), clearly demonstrates that the substrate specificity of PTP-FEST is highly restricted.

Many intracellular PTPs are limited in their substrate availability due to strict confinement within a particular subcellular location; examples include FTF1B, which is localized to the cytoplasmic face of the endoplasmic reticulum (Frangioni et al., Cell 68:545-560 (1992)), and TCPTP which is either nuclear (Tillmann et al., Mol. Cell. Biol. 14:3030-3040 (1994)) or localized to the endoplasmic reticulum, depending upon which alternative spliced form is expressed (Lorenzen et al., J. Cell Biol. 131:631-643 (1995)). Alternatively, certain PTPs appear to be highly regulated, requiring activation before appreciable activity

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can be demonstrated. For example, the SH2 domain-containing PTPs, SHP1 and SHP2, display relatively low activity in vitro, but can be considerably activated by several mechanisms including C-terminal truncation (Zhao et al., J. Biol. Chem. 268:2816-2820 (1993)), addition of certain phospholipids (Zhao et al., Proc. Natl. Acad. Sci. USA 90:4251-4255 (1993)), or SH2 domain-mediated binding of appropriate phosphotyrosine-containing peptides (Lechleider et al., J. Biol. Chem. 268:21478-21481 (1993)).

10        However, PTP-PEST exhibits high specific activity in vitro (35,000 U/mg), and is a predominantly (90-95%) soluble PTP within cells (Garton and Tonks, unpublished data); in principle, therefore, it may act potently on any substrate accessible to the cytoplasm. This accessibility may partly  
15        underlie the necessity for PTP-PEST to possess an inherently constrained substrate specificity. The demonstration that mutant PTP-PEST is capable of exclusively associating with p130<sup>cas</sup> in an intracellular context in the presence of many other tyrosine phosphorylated proteins, is an indication  
20        that the narrow substrate specificity of the enzyme may result in PTP-PEST having a negligible influence on the phosphorylation state of the majority of tyrosine phosphorylated proteins within the cell, even though those substrates are largely accessible to PTP-PEST.

25        The role of p130<sup>cas</sup> in cellular transformation by the v-crk and v-src oncogenes is unclear, although there is a general correlation between the level of tyrosine phosphorylation of p130<sup>cas</sup> and the degree of transformation in cells expressing different forms of crk or src (Kanner et al., EMBO J. 10:1689-1698 (1991); Mayer and Hanafusa, J. Virol. 64:3581-3589 (1990)). Furthermore, enhanced tyrosine phosphorylation of p130<sup>cas</sup> has also been observed in cells transformed by c-Ha-ras and by ornithine decarboxylase overexpression (Auvinen et al., Mol. Cell. Biol. 15:6513-  
30        6525 (1995)). Expression of antisense cDNA encoding  
35



p130<sup>cas</sup> in these cells results in a partial reversion of the transformed phenotype. These observations suggest that aberrant tyrosine phosphorylation of p130<sup>cas</sup> is a common feature of cells transformed by several disparate mechanisms and that p130<sup>cas</sup> may be required for full manifestation of the transformed state. Dephosphorylation of p130<sup>cas</sup> by PTP-PEST is therefore a potentially important regulatory mechanism for counteracting the transforming effects of various oncogenes.

10 Tyrosine phosphorylation of p130<sup>cas</sup> has been observed in fibroblasts following integrin-mediated cell adhesion to extracellular matrix proteins (Nojima et al., J. Biol. Chem. 270:15398-15402 (1995); Petch et al., J. Cell Science 108:1371-1379 (1995); Vuori and Ruoslahti, J. Biol. Chem. 270:22259-22262 (1995)). Under these conditions, using an antibody (4F4) that predominantly recognizes tyrosine phosphorylated p130<sup>cas</sup> (Kanner et al., EMBO J. 10:1689-1698 (1991); Petch et al., J. Cell Science 108:1371-1379 (1995)) it was shown that phosphorylated p130<sup>cas</sup> is localized to  
15 20 focal adhesions (Petch et al., J. Cell Science 108:1371-1379 (1995)), whereas fractionation studies have demonstrated that the normal cellular location of the majority of non-phosphorylated p130<sup>cas</sup> is the cytosol (Sakai et al., EMBO J. 13:3748-3756 (1994)). Furthermore, in crk-transformed  
25 fibroblasts, tyrosine phosphorylated p130<sup>cas</sup> is detected only in insoluble fractions (Sakai et al., EMBO J. 13:3748-3756 (1994)), suggesting that both cell adhesion- and transformation-mediated phosphorylation of p130<sup>cas</sup> is associated with redistribution of the protein from the  
30 cytosol to focal adhesions.

It is plausible that the redistribution of tyrosine phosphorylated p130<sup>cas</sup> may be driven by its association with FAK, which is constitutively associated with focal adhesions due to its C-terminal focal adhesion targeting  
35 domain (Hildebrand et al., J. Cell Biol. 123:993-1005

(1993); Schaller et al., Proc. Natl. Acad. Sci. USA 89:5192-5196 (1992)). The sequestration of tyrosine phosphorylated p130<sup>cas</sup> in focal adhesions both in transformed cells, and following integrin-mediated cell adhesion, strongly suggests a role for p130<sup>cas</sup> in signalling events in this region of the cell. One consequence of the redistribution of tyrosine phosphorylated p130<sup>cas</sup> is likely to be that, in addition to localizing p130<sup>cas</sup> to a region of the cell containing abundant protein tyrosine kinase activity, the phosphorylated protein will be relatively inaccessible to the cytosolic phosphatase PTP-PEST. This raises the possibility that the role of PTP-PEST in dephosphorylating p130<sup>cas</sup> may be to prevent inappropriate tyrosine phosphorylation of the cytosolic pool of p130<sup>cas</sup>, thus preventing formation of signalling complexes assembled around tyrosine phosphorylated p130<sup>cas</sup> in inappropriate cellular locations.

Several mitogenic factors potently stimulate tyrosine phosphorylation of p130<sup>cas</sup>. These include agents acting through heterotrimeric G protein-coupled receptors such as lysophosphatidic acid (Seufferlein and Rozengurt, J. Biol. Chem. 269:9345-9351 (1994)), bradykinin (Leeb-Lundberg et al., J. Biol. Chem. 269: 24328-24344 (1994)), and bombesin (Zachary et al., J. Biol. Chem. 267:19031-19034 (1992)), as well as growth factors that activate receptor tyrosine kinases, namely PDGF (Rankin and Rozengurt, J. Biol. Chem. 269:704-710 (1994)), EGF and NGF (Ribon and Saltiel, J. Biol. Chem. 271:7375-7380 (1996)). These observations suggest roles for p130<sup>cas</sup> in regulation of mitogenic signalling pathways, presumably involving assembly of signalling complexes based on tyrosine phosphorylated p130<sup>cas</sup>. The identities of the proteins involved in these complexes are not established, but are likely to include SH2 domain containing adaptor proteins such as crk (Ribon and Saltiel, J. Biol. Chem. 271:7375-7380 (1996)), and its

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associated proteins (Feller et al., Oncogene 10:1465-1473 (1995); Hasegawa et al., Mol. Cell. Biol. 16:1770-1776 (1996); Knudsen et al., J. Biol. Chem. 269:32781-32787 (1994); Matsuda et al., Mol. Cell. Biol. 14: 5495-5500 (1994); Tanaka et al., Proc. Natl. Acad. Sci. USA 91:3443-3447 (1994)). Therefore tyrosine phosphorylation and dephosphorylation of p130<sup>cas</sup> potentially plays a central role in regulating the formation of such complexes, thereby influencing downstream events in mitogenic signalling.

10 Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

CLAIMS

We claim:

1. A protein tyrosine phosphatase wherein the invariant  
aspartate residue is replaced with an amino acid (e.g.  
5 alanine) which:
  - a) does not cause significant alteration of the  $K_m$   
of the enzyme but which results in a reduction in  
10  $K_{cat}$  to less than 1 per minute; or
  - b) is selected from the group consisting of:  
alanine, valine, leucine, isoleucine, proline,  
15 phenylalanine, tryptophan, methionine, glycine,  
serine, threonine, cysteine, tyrosine, glutamine,  
lysine, arginine and histidine, and wherein the  
phosphatase binds to a tyrosine phosphorylated  
substrate and is catalytically attenuated.
2. A protein tyrosine phosphatase according to claim 1(a)  
which is selected from the group consisting of: PTP1B  
(and wherein for example the invariant aspartate  
20 residue is located at position 181), PTP-PEST (and  
wherein for example the invariant aspartate residue is  
located at position 199), PTP $\tau$ , MKP-1, DEP-1,  
PTP $\mu$ , PTPX1, PTPX10 and PTPH1.
3. A protein tyrosine phosphatase according to claim 1(b)  
which is a PTP-PEST phosphatase in which the amino  
25 acid at position 231 is replaced with a serine  
residue.
4. A method of identifying a tyrosine phosphorylated  
protein which is a substrate of a protein tyrosine  
phosphatase, comprising the steps of:  
30 a) combining at least one tyrosine phosphorylated  
protein with at least one protein tyrosine

phosphatase in which the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute, under conditions appropriate for formation of a complex between the tyrosine phosphorylated protein and the protein tyrosine phosphatase, thereby producing a combination; and

10      b) determining the presence of absence of a complex in the combination wherein the presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the protein tyrosine phosphatase with

15      which it forms a complex.

5.      A method according to claim 4, wherein the protein tyrosine phosphatase is as defined in any one of claims 1-3.
6.      A method according to claim 4 or claim 5 wherein the tyrosine phosphorylated protein is selected from the
- 20      group consisting of: p130<sup>cas</sup>, the EGF receptor, p210 bcr:abl, MAP kinase and the insulin receptor.
7.      A kit for identifying a tyrosine phosphorylated protein substrate of a protein tyrosine phosphatase
- 25      comprising:
- a)      at least one protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which does not cause significant alteration of the  $K_m$  of the
- 30      enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute; and

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- b) ancillary reagents suitable for use in detecting the presence or absence of a complex between the protein tyrosine phosphatase and a tyrosine phosphorylated protein,  
5 wherein for example the protein tyrosine phosphatase is as defined in any one of claims 1-3.
8. A method of identifying an agent which alters the interaction between a protein tyrosine phosphatase and a tyrosine phosphatase, comprising the steps of:
- 10 a) identifying a tyrosine phosphorylated protein which is a substrate of a protein tyrosine phosphatase;
- b) combining the tyrosine phosphorylated protein and the protein tyrosine phosphatase and an agent to  
15 be tested under conditions suitable for interaction between the tyrosine phosphorylated protein and the protein tyrosine phosphatase, thereby forming a combination;
- c) determining the amount of enzymatic activity in  
20 the combination; and
- d) comparing the amount of enzymatic activity determined in (c) with the amount of enzymatic activity in the absence of the agent to be tested, under conditions suitable for interaction  
25 between the tyrosine phosphorylated protein and the protein tyrosine phosphatase,  
wherein a difference in the enzymatic activity indicates that the agent alters the interaction between the protein tyrosine phosphatase and the  
30 tyrosine phosphorylated protein.
9. A method of identifying an agent which alters the interaction between a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate

of the protein tyrosine phosphatase, comprising the steps of:

- 5 a) identifying a tyrosine phosphorylated protein which is a substrate of a protein tyrosine phosphatase;
- b) combining the tyrosine phosphorylated protein, a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which does not cause  
10 significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute, and an agent to be tested, under conditions suitable for interaction between the tyrosine phosphorylated protein and the  
15 protein tyrosine phosphatase, thereby forming a combination;
- c) determining the extent of binding between the tyrosine phosphorylated protein and the protein tyrosine phosphatase in the combination; and
- 20 d) comparing the extent of binding determined in (c) with the extent of binding in the absence of the agent to be tested, under conditions suitable for interaction between the tyrosine phosphorylated protein and the protein tyrosine phosphatase,  
25 wherein a difference in the extent of binding indicates that the agent alters the interaction between the protein tyrosine phosphatase and the tyrosine phosphatase and the tyrosine phosphorylated protein.

30 10. A method according to claim 8 or claim 9 wherein if the amount of enzymatic activity or the extent of binding, respectively, is:

- a) greater in the presence of the agent to be tested than in the absence of the agent, then the agent

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enhances the interaction between the protein tyrosine phosphatase and the tyrosine phosphorylated protein; or

- 5           b)   less in the presence of the agent to be tested than in the absence of the agent, then the agent inhibits the interaction between the protein tyrosine phosphatase and the tyrosine phosphorylated protein.

10       11.   A protein tyrosine phosphatase (e.g. as defined in any one of claims 1-3) for use in therapy, prophylaxis or diagnosis, for example in:

- a)   the treatment of conditions in which a reduction in the activity of a tyrosine phosphorylated protein is indicted; and/or
- 15       b)   reducing the activity of a tyrosine phosphorylated protein; and/or
- c)   a method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute, whereby formation of the complex reduces the activity of the tyrosine phosphorylated protein; and/or
- 20       d)   the treatment of conditions in which a reduction in the transforming effects of oncogenes associated with  $p130^{cas}$  phosphorylation is indicated; and/or
- 25       e)   reducing the transforming effects of oncogenes associated with  $p130^{cas}$  phosphorylation; and/or
- 30       f)   a method of reducing the transforming effects of oncogenes associated with  $p130^{cas}$  phosphorylation



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- comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates p130<sup>cas</sup> and reducing the transforming effects of oncogenes associated with p130<sup>cas</sup> phosphorylation; and/or
- g) the treatment, therapy, diagnosis or prophylaxis of cancer (for example cancers associated with p130<sup>cas</sup> phosphorylation); and/or
- h) the treatment of conditions associated with oncogenic activity (e.g. with v-crk, v-src and/or c-Ha-ras activity); and/or
- i) the treatment of conditions in which a reduction in the formation of signalling complexes associated with p130<sup>cas</sup> is indicated; and/or
- j) reducing the formation of signalling complexes associated with p130<sup>cas</sup>; and/or
- k) a method of reducing the formation of signalling complexes associated with p130<sup>cas</sup> comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates p130<sup>cas</sup> and reducing the formation of signalling complexes associated with p130<sup>cas</sup>, and/or

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- 1) the prevention of the induction of mitogenic pathways;
  - m) the treatment of conditions in which the prevention of the induction of mitogenic pathways is indicated;
  - 5 n) the treatment of conditions in which a reduction in the cytotoxic effects associated with protein tyrosine phosphatase administration or over-expression is indicated; and/or
  - 10 o) reducing the cytotoxic effects associated with protein tyrosine phosphatase administration or over-expression; and/or
  - p) a method for reducing the cytotoxic effects associated with protein tyrosine phosphatase administration or over-expression comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute, in place of a corresponding wild type protein tyrosine phosphatase; and/or
  - 20 q) in a method according to any one of claims 13-20.
- 25 12. Use of a protein tyrosine phosphatase for the manufacture of a medicament for treatment, prophylaxis or diagnosis (for example for use in the treatments defined in claim 11).
- 30 13. A method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid which does not cause significant

alteration of the  $K_m$  of the enzyme but which results in a reduction in  $K_{cat}$  to less than 1 per minute, whereby formation of the complex reduces the activity of the tyrosine phosphorylated protein.

- 5 14. A method according to claim 13, wherein the tyrosine phosphorylated protein is selected from the group consisting of: p130<sup>cas</sup>, the EGF receptor, p210 bcr:abl, MAP kinase and the insulin receptor.
- 10 15. a method according to claim 13, wherein the protein tyrosine phosphatase is selected from the group consisting of: PTP1B, PTP-PEST, PTP $\gamma$ , MKP-1, DEP-1, PTP $\beta$ , FTX1, FTX10 AND PTPH1.
- 15 16. A method of reducing the transforming effects of oncogenes associated with p130<sup>cas</sup> phosphorylation comprising of administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the  $K_m$  of the enzyme but  
20 which results in a reduction in  $K_{cat}$  to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates p 130<sup>cas</sup>, thereby negatively regulating the downstream effects of p130<sup>cas</sup> and reducing the transforming effects of oncogenes associated with  
25 p130<sup>cas</sup> phosphorylation.
17. A method according to claim 16, wherein the oncogene is selected from the group consisting of: v-erbB, v-src and c-Ha-ras.

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18. A method of reducing formation of signalling complexes associated with p130<sup>cas</sup> comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST OR PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates p130<sup>cas</sup>, thereby negatively regulating the downstream effects of p130<sup>cas</sup> and reducing the formation of signalling complexes associated with p130<sup>cas</sup>.
19. A method according to claim 18, which prevents the induction of mitogenic pathways.
20. A method of reducing cytotoxic effects associated with protein tyrosine phosphatase administration or over expression, comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, in place of a corresponding wild type protein tyrosine phosphatase.

	70	80	90	100	110	120	130
PTP1B	DYINASLI	KMEEAQRSYILTQGPLNPNTCGHFWEMVWE	QKSRGVVMLNRVM				
CTPP	DYINASLV	DIEEAQRSYILTQGPLNTPCCHFWMVWQ	QKTAKAVMLNRIV				
PTP_xi_D1	DGKLTDYINANV	DGYN RPKAYIAAQGPLKSTAEDEFWRMIWE	HNVFIVMITNLV				
PTP_zela_D1	DGKLTDYINANV	DGYN RPKAYIAAQGPLKSTAEDEFWRMIWE	HNVFIVMITNLV				
PTP_gamma_D	DSKHSDYINANV	DGYN KAKAYIATQGGLKSTFEDEFWRMIWE	QNTGIIVMITNLV				
PTP99A_D1	KKNL DYINANFI	DGYR KGHAFIGTQGGLPDFTDGFCDWRMIWE	QRTAIIVMITNLV				
LCA_D1	PGS DYINANFI	DGYR KQNAHYIATQGGLPETMTGDFWRMVWE	QRTATVMMTRLE				
PTP_mu_D1	TNS DYINGNYI	DGYH RPNHYIATQGPQMETIYDFWRMVWH	ENTASIIIMVTNLV				
PTP_alpha_D1	PDS DYINASFI	NGYQ EKNKFIAAQGPKEETVNDFFWRMIWE	QNTATIIVMTNLK				
PTP_opsilon_D	PCS DYINASFI	DGYK EKNKFIAAQGPKEETVNDFFWRMIWE	QKSATIVMLTNLK				
so_CD45_D1	AGS TYINASFI	DGFK EPRKYIAAQGPRDETVDFFWRMIWE	QKATIVIMVTRCE				
SH_PTP2	EPV SDYINANIIIMPEFETKCNNSK	PKKSYIATQGCLQNTVNDFFWRMVFG	ENSRVIVMTTKEV				
SH_PTP1	IPG SDYINANVIKQNLL	GPDE NAKTYIASQGCLCATVNDFFWQMAWQ	ENSRVIVMTTREV				
PTP_bola	PCS DYINASFI	PGNN FRREYIVTQGLPGTKDDFWKMVWE	QNVHNIVMTTCOV				
s_PTP10D	EGS DYINANV	PGHN SPREFIATQGGLPHSTRDDFWRMVWE	SNSRAIVMLTRCF				
SAP_1	PGS DYINASFM	PGLW SQEFIATQGGLPQTGDFWRLVWE	QQSHTLVMLTNCM				
PTP_STEP	DPL SSYINANFI	RGYNGEEKVYIATGGPIVSTVDFWRMVWQ	ERTPIIVMITNIE				
s_PTP69A_D1	QTT DYINANFV	IGYK ERKKFICACGPMESTIDDFFWRMIWE	QHLEIIVILTNLE				
MEG2	TQT DYINASFM	DGYK QKNAYIGTGPLENTYRDDFWLMVWE	QKVLVIVMTTRFE				
PTP_PEST	QDS DYINANFI	KGVY GPKAYVATGGPLANTVIDFWRMVWE	YNVSIIVMACREF				
PTPH1	INA SYVNMEI	PAAN LVNKYIATQGGLPHTCIAQFWQVVWD	QKLSIIVMLTTLT				
PTP1	EGS DYINANFI	DGAY PKOFICTQGGLPNTIADFFWRMVWE	NRCRIIVMLSRES				
ss_yeast_pyp1	EL DYINASFI	KTETSNIYACQGISRSISDFWFMVNDVENIGTIIVMLGSLE	ENIGTIIVMLGSLE				
ss_yeast_pyp2	S DYINASHI	DVGNNKIYACQAPKPGTLDDFFEMVWNHNSGTNGVIVMLTNLY	HNVEVI VMITNLV				
a_PTP_xi_D2	DGKLTDYINANV	DGYN RPKAYIAAQGPLKSTAEDEFWRMIWE	HNSTII VMLTKLR				
a_LCA_D2	EGS DYINASFL	DGYR QQKAYIATQGFLAESTEDFWRLWE	WKSCSI VMLTELE				
a_PTP_alpha_D2	ENT DYVNASFI	DGYR QKDSYIASQGFLHTIEDFWRMWE	WKSHITI VMLTEVQ				
a_PTP_opsilon_D2	EYT DYINASFI	DGYR QKDYFIATQGFLAHTVEDFWRMWE	YHCTSIVMLNDVD				
a_PTP_mu_D2	SS NYINAALM	DSYK QPSAFIVTGHPLPNTVKDFWRLVLD	RKVKVI VMLTELVD				
ase_CD45_D2	SEETSKYINASFV	MSYW KPEmmaIAAQGPLKETIGDFWQMFQ	QSVTILVMISEIG				
os_PTP69A_D2	ENS TYINASFI	ESGD NSETFIIAQDPFENTIGDFWRMISE	HNAQLVMIIPDGQ				
a_PTP_zela_D2	GT DYINASFI	MGYQ QSNEFIITGHPLLHTTKDFWRMWD	HNAQII VMLPDNQ				
a_PTP_gamma_D2	KGT DYINASFI	MGYQ RSNEFIITGHPLPHTTKDFWRMWD	HNAOTVVLSSLD				
os_PTP99A_D2	GEDGSDYINASWL	HGFR RLDRDFIVTGHFMAHTIKDFWQMVWD	NRTIPVLAVALSSS				
rsinia_PTP	NYIOVG	NRTIACQYPLOSOESHFERMLAE	NRTIPVLAVALSSS				
p1Bseq.no.	70	80	90	100	110	120	130

↑<sub>2</sub>

2

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Hum_PTP1B	1	DFPCRVAKLKPKN	20	RNRYDVSPFDHSRIKLHQE	30	50	60	DN
Hum_TCPTP		DYPHRVAKFPENR		RNRYDVSPYDHSRV LQNA				EN
Hum_PTP_xi_D1		GITADSSNHPDNKH		KNRYINIVAYDHSRVKLAQL				AEK
Hum_PTP_zela_D1		GITADSSNHPDNKH		KNRYINIVAYDHSRVKLAQL				AEK
Hum_PTP_gamma_D		NITAEHSNHPDNKH		KNRYINILAYDHSRVKLRPL				PGK
Dros_PTP99A_D1		DLPCHEHSQHPENR		KNRYLNITAYDHSRVHLHPT				PGQ
Hum_LCA_D1		QFTWENSNLEVNKP		KNRYANVIAYDHSRVILTSI				DGV
Hum_PTP_mu_D1		SAPWDSAKKDNRM		KNRYGNIILAYDHSRVRLQTI				EGD
Hum_PTP_alpha_D1		QATCEAASKEENKE		KNRYVNILPYDHSRVHLTPV				EGV
Hum_PTP_opsilon_D		QGTFFELANKENRE		KNRYPNILPNDHSRVILSQL				DGI
Mouso_CD45_D1		KFPIKDARKPHNQ		KNRYVDILPYDYNRVELSEI				NGD
Hum_SH.PTP2		LYSRKEGQOENKN		KNRYKNILPFDHTRVVLHDG				DPN
Hum_SH.PTP1		LHQRLGQRPENKG		KNRYKNILPFDHSRVILQGR				DSN
Hum_PTP_bola		NQSCDIALLPENRG		KNRYNNILPYDATRVKLSNV				DDD
Dros_PTP10D		DQPCFTADLPCNRP		KNRFTNILPYDHSREFKLQPV				HEE
Hum_SAP.1		SQSOMVASASENNA		KNRYRNVLPYDWSRVPLKPI				DPE
Ral_PTP_STEP		FVDPKEYDIPGLVR		KNRYKTILPNPHSRVRLTSP				NGL
Dros_PTP69A_D1		DRTTKNSDLKENAC		KNRYPDIKAYDQTRVKLAVI				SGH
Hum_MEG2		VGTFHCMSMSPGNLE		KNRYGDVPCLDQTRVKLTKR				TPS
Hum_PTP.PEST		IYPTATGEKEENVK		KNRYKDILPFDHSRVKLTLLK				EDY
Hum_PTPH1		GLAITFAKLQNLD		KNRYKDVLPYDTRVLLQGN				QDK
Dici_PTP1		PSETSEGDKKHNTS		KNRYTNILPVNHTRVQLKKI				SPS
Fiss_yeast_pyp1		QWSTVDLSLNTSYK		KNRYTDIVPYNCTRVHLKRT				KGC
Fiss_yeast_pyp2		WCCLASSRSTSISSR		KNRYTDIVPYDKTRVRLAVP				AEK
Hum_PTP_xi_D2		GITADSSNHPDNKH		KNRYINIVAYDHSRVKLAQL				RGV
Hum_LCA_D2		TSRFISANLPCNKF		KNRLVNIMPYELTRVCLQPI				RGE
Hum_PTP_alpha_D2		NDKMRTGNLPCNMF		KNRVLQIIPYEFNRRVILPVK				RGQ
Hum_PTP_opsilon_D2		KENMRTGNLPCNMF		KARVIOIIPYDFNRRVILSMK				DGE
Hum_PTP_mu_D2		VEDCSIALLPNHE		KNRCDILPPDRCCLPFLITI				PMR
Mouse_CD45_D2		WRTQHIGNQENKK		KNRNSNVVPYDFNRRVPLKHELEMSKESEPESESSDDSD				SGE
Dros_PTP69A_D2		SKSCSVGENEENNM		KNRSQEIIPYDNRVILTP				PGM
Hum_PTP_zeia_D2		QSDYSAALKQCNR		KNRTSSIIIPVERSRVGISSL				
Hum_PTP_gamma_D2		VECFSAQKECNKE		KNRNSVVPSEARVGLAPL				
Dros_PTP99A_D2		ETNLMAEOVEELKNCTPYLEQQKNIIQFOPKDIHISAMKOVNSIKNRGALFPIEGSRVHLTPKP						
Yarsinia_PTP		TNDPRYLOACGGEKI		LNRRFDIOCCROTAVRAD				
PTP1Bseq.no.		30	40	50	60			

PTP1B66

Figure 1B

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Hum_PTP1B	140	EKGLKCA	QYWPQKEEM	150	IFEDTNLKLTLISEDIKSYTVLELE	160	170	180	190	200
Hum_ICPTB		EKESVKA	QYWPT DDQEM		LFKETGFSVKLLSEDVKSYYTVLQLE					NLTQTETREI
Hum_PTP_xi_D1		EKGRRKCD	QYWPP ADGSE		EYGN FLVTQKSVQVLAYTVFTLRNTKIKKG					NINSGETRTI
Hum_PTP_zela_D1		EKGRRKCD	QYWP ADGSE		EYGN FLVTQKSVQVLAYTVFTLRNTKIKKG					SQGRPSGRV
Hum_PTP_gamma_D		EKGRRKCD	QYWP TENSE		EYGN IIVTLKSTKIHCYTVFSIRNTKVKKGQKGNPKGRQNERV					SQGRPSGRV
Dros_PTP99A_D1		ERGRVKCD	MYWP KDGE		EYGN IQVKLIEEVMSSTVTLQIKHLKLKK					KKQCNTKLV
Hum_LCA_D1		EKSRVKCD	QYWP ARGTE		TCGL IQVTLDTVELATYTVFALH					KSGSEKREL
Hum_PTP_mu_D1		EVGRVKCC	KYWP DDTE		IKVTLIETELLAAYVIFAVE					KRGVHEIREI
Hum_PTP_alpha_D1		ERKECKCA	QYWP DQGW		TYGN IRSVEDVTVLVDYTVFCIQQVG					DMTNRPQRLI
Hum_PTP_opsilon_D		ERKECKCH	QYWP DQGW		TYGN IRVCVEDCVLVLYTIFCIQPOL					PDGCKAPRLV
Hum_CD45_D1		EGNRNKCA	EYWPMEEGTR		AFKD IIVTINDHKRCPDYIILNVAH					KKEKATGREV
Hum_SH_PTP2		ERGKSKCV	KYWP EYALK		EYGV MRVRNVKESAAHDYTLKLKSK					VGQNTERTV
Hum_SH_PTP1		EKGRNKCV	PYWP VGMQR		AYGP YSVTNGGEHDTTEYKLLQVSP					LDNGDLIREI
Hum_PTP_bola		EKGRVKCD	HYWP DQDSL		YYPD LILQMLSESVPLEWTIFKICG					EEQLDAHRLI
Dros_PTP10D		EKGREKCD	HYWPL DSQPC		YFGD IKVQILNDSHYADWVMEMLC					RGSEQRIL
Hum_SAP.1		EAGRVKCE	HYWPL DSQPC		THGH LRVTLVGEEVMEVNTVLLLL					QVEEQKTLV
Ral_PTP_STEP		EMN EKCT	EYWP EEQV		VHDG VEITVQKVIHTEDYRLISLR					RGTEERGL
Dros_PTP69A_D1		EYNKAKCA	KYWPKEVFTK		QFGD ILVKFAQERKTGDYIELNVSKNAN					VGEEEDRRQI
Hum_MEG2		EGGRKCKG	QYWPLEKDSRI		RFGF LTVTNLGVENNMHYKLEIH					NTEERQKRQV
Hum_PTP_PEST		EMGRKKCE	RYWPLYGEDPI		TFAP FKISCEDEQARTDYFILLLE					FQNESRRL
Hum_PTPH1		ERGRTKCH	QYWP PPDMV		NHGG FHIQCQSEDCTIAYVSMVLT					NTQTGEEHTV
Dici_PTP1		ENCRICKD	RYWPEQIGGEQFSIYGN		VEFGTYSVELVEVIOCREIITRNIR					LTFEGETRDI
Fiss_yeast_ptp1		EAGREMCT	AYWPSNGIGDK		QYVGYCYVKQISENVDSRFLFEIQ					NANFPSVKV
Fiss_yeast_ptp2		EAGSEKCS	QYWPDKDHALCEGG		LRISVOKYETEFEDLVHLRL					DKPNGPFXI
Hum_PTP_xi_D2		EKGRRKCD	QYWP ADGSE		EYGN FLVTQKSVQVLAYTVFTLRNTKIKKG					SQGRPSGRV
Hum_LCA_D2		EMGREKCH	QYWP AERSA		RYQY FVVDPMAYNMPQYILFKVT					DARDGQSRTI
Hum_PTP_alpha_D2		ERGQEKCA	QYWP SDGLV		SYGD ITVELKKEEESYTVLLVT					NTRENKSRQI
Hum_PTP_opsilon_D2		EREQDKCY	QYWP TEGSV		THGE ITIEIKNDTLSEALISFLVTINQPO					ARQEEQVRV
Hum_PTP_mu_D2		PA QLCP	QYWP ENGVH		RHGP IQVEFVSADLEEDIIISFRIYNA					ARPQDGYRMV
Mouse_CD45_D2		NGDQEVCA	QYWP GEGKQ		TYGD MEVEMKDTNRSAYTLFELR					HSKRKEPRTV
Dros_PTP69A_D2		D GPRKCP	RYWA DDEVQ		YDH ILVKVHSESCTPYTFYVT					NCKIDDTLKV
Hum_PTP_zela_D2		NMAEDEFV	YWPNKDEPINCESFKVTL		MAEEHKCLSNEEKLIIIFILE					ATQDDYVLEV
Hum_PTP_gamma_D2		SLAEDEFV	YWP SREESMNCFAFTVTL		SKDRCLCSNEEQIIIFILE					ATQDDYVLEV
Dros_PTP99A_D2		D INFA	QFWDPEATPIPSDHY		RVKFLNKTNKSQDVSEVIO					SIODDYELTV
Yarsinia_PTP		ETANOREGMPDYFR	OSGT YGSITVESKMTQOVGL		GDGINWYTLTI					REAGOKT.SV
PTP1Bseq.no.										

PTP1B66

β5

β6

β7

β8

β6 Figure 1C

Hum_PTP1B	LHFHYTTWPDF	G	VPESPASFLNFLFKVRES	GSLSPEHG	PVVVHCSAGIGRS	GTFC	270
Hum_TCPTP	SHFHYTTWPDF	G	VPESPASFLNFLFKVRES	GSLNPDHG	PAVHCSAGIGRS	GTFS	265
Hum_PTP_xi_D1	TQYHYTQWPDF	G	VPEYSLPVLTFVRKAAYA	KRH AVG	PVVVHCSAGVGR	TGYI	260
Hum_PTP_zela_D1	TQYHYTQWPDF	G	VPEYSLPVLTFVRKAAYA	KRH AVG	PVVVHCSAGVGR	TGYI	255
Hum_PTP_gamma_D	IQYHYTQWPDF	G	VPEYALPVLTFVRRSSAA	RMP ETG	PVLVHCSAGVGR	TGYI	250
Dros_PTP99A_D1	YQYHYTNWPDF	G	TPDHPPLVNLNFVKSSAA	NPA EAG	PIVVHCSAGVGR	TGYI	245
Hum_LCA_D1	RQFQFMAWPDF	G	VPEYPTPIAFLRRVKAC	NPL DAG	PMVVHCSAGVGR	TGCFI	240
Hum_PTP_mu_D1	RQFHFTGPDF	G	VPHATGLLGFVRQVSK	SPP SAG	PLVVHCSAGVGR	TGCFI	235
Hum_PTP_alpha_D1	TQFHFTSPDF	G	VPFTPIGMLKFLKKVKT	NPQ YAG	AIVVHCSAGVGR	TGTFV	230
Hum_PTP_opsilon_D	SQLHFTSPDF	G	VPFTPIGMLKFLKKVKT	NPV HAG	PIVVHCSAGVGR	TGTFI	225
Mouso_CD45_D1	THIQFTSPDF	G	VPEDPHLLKLRNRVNAF	SNF FSG	PIVVHCSAGVGR	TGTFI	220
Hum_SH.PTP2	WQYHFTWPDF	G	VPSPDGGVLDLLEEVHK	QESIMDAG	PVVVHCSAGIGR	TGTFI	215
Hum_SH.PTP1	WHYQYLSWPDF	G	VPSEPGVLSFLDQINQR	QESLPHAG	PIIVHCSAGIGR	TGTFI	210
Hum_PTP_bola	RHFHYTVWPDF	G	VPETQSLIQFVRTVRDY	INRSPGAG	PTVVHCSAGVGR	TGTFI	205
Dros_PTP10D	RHFHTTWPDF	G	VPNPPQTLVRFVRAFRDR	ICA EQR	PIVVHCSAGVGR	SRTGTFI	200
Hum_SAP.1	RQFHYQAWPDF	G	VPSSPDTLAFLWRMLRQW	LDQTMEGG	PPIVHCSAGVGR	TGTLI	195
Ral_PTP_STEP	KHYWFTSWPDF	K	TPDRAPPLHLVREVEEAA	QQEGPHCS	PIIVHCSAGIGR	TGCFI	190
Dros_PTP69A_D1	TQHYLTWPDF	M	APHPHGIKFIQINSVSLQ	RG	PILVHCSAGVGR	TGTLV	185
Hum_MEG2	THFQFLSWPDF	G	VPSSAASLIDFLRVNRQSL	AVSNMGARSKGQC	PEPPIVVHCSAG	IGRTGTFC	180
Hum_PTP.PEST	YQFHYVNWPDF	D	VPSSFDLIDMISLMRKQEH	DV	PICIHCSAGCGRT	GAIC	175
Hum_PTPH1	THLQYVAVPDF	G	IPDDSSDFLEFVNVRSLRVDSE		PVLVHCSAGIGR	TGVLV	170
Dici_PTP1	TQYQYEGWPDF	N	IPDHTQFPRLHLSITNRQN	QIIPSSD	RNVPIIVHCSAG	VGRGTGFC	165
Fiss_yeast_pyp1	HHYQYPNWSDC	N	SPENVKSMVEFLKVNNSHSG		NT-VHCSAGVGR	TGTFI	160
Fiss_yeast_pyp2	HHFWHTWED	K	THPDIESILGLRCDIKVPNDG		PMFVHCSAGVGR	TGTFI	155
Hum_PTP_xi_D2	TQYHYTQWPDF	G	VPEYSLPVLTFVRKAAYA	KRH AVG	PVVVHCSAGVGR	TGTYI	150
Hum_LCA_D2	RQFQFTDWPEQ	G	VPKTGEFIDFQVHKT	KEQFGQDG	PIVVHCSAGVGR	TGVFI	145
Hum_PTP_alpha_D2	RQFHFGWPEV	G	IPSDGKGMISIIAAVQKQ	QQQ SGNH	PIVVHCSAGAGRT	GTFC	140
Hum_PTP_opsilon_D2	RQFHFGWPEI	G	IPAEKGMDILIAAVQKQ	QQQ TGNH	PIVVHCSAGAGRT	GTFI	135
Hum_PTP_mu_D2	QQFQFLGWPMYRD	TPVSKRSFLKLRQVDK	WQEEYNGEG		PTVVHCLNGGGRS	GTFC	130
Mouse_CD45_D2	YQYQCTTWKGE	E	LPAEPKDLVSMIQDLKQL	PKASPEGMKYH	KHASILVHCRDGS	QQQTGLFC	125
Dros_PTP69A_D2	TQFQYNGWPTVDGE	VEVCGI	IELVDQAYNHYNKNSGC		RSPLTVHCSLGT	DRSSIFV	120
Hum_PTP_zeia_D2	RHFQCPKWPN	PDSPISKT	FELISVKEEAANR	DG	PMIVHDEHGVTA	GTFC	115
Hum_PTP_gamma_D2	RHFQCPKWPN	PDAPISST	FELINVIKEEALTR	DG	PTIVHDEYGA	VSAGMLC	110
Dros_PTP99A_D2	KMLHCPSWPEM	SNPNSIYDF	FIYDVHERCNDY	RNG	PIVIVDRYGAQA	CTFC	105
Yarsinia_PTP	PVVHVGNWPDOTAVS	SEVTKALASLVD	QTAETKRNMYE	SKSSAVADD	SKLRPIVHCRAG	VGRTAOLI	100
PTP1Bseq.no.							95



Figure 1D



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	280	290	300	310	320	330	340
PTP1B	LADTCLLLMDKR	KDPSSVDI	KKVLLMRKFRMG	LIQADQLRFSYLAVIEGAKFIMGD			
TCPTP	LVDTCVLVMEKG	DD	INI	KQVLLNMRKYRMG	LIQTPDQLRFSYMAIEGAKCIKGDSS		
PTP_xi_D1	VLDMLQIQIHE	GT	VNI	FGFLKHRSQRNY	LVQTEEQYVFIHDTLVEAILSKETEV		
PTP_zela_D1	VLDMLQIQIHE	GT	VNI	FGFLKHRSQRNY	LVQTEEQYVFIHDTLVEAILSKETEV		
PTP_gamma_D	VLDMLQIQIKOK	ST	VNV	LGFLKHRTQRNY	LVQTEEQYVFIHDTLVEAILSKETEV		
PTP99A_D1	VLDAMLKQIQK	NI	VNV	FGFLRHRAQRNF	LVQTEEQYVFIHDTLVEAILSKETEV		
LCA_D1	VIDAMLERMKHE	KT	VDI	YGHVTCMRQRNY	MVQTEEQYVFIHDTLVEAILSKETEV		
PTP_mu_D1	VIDIMLDMARE	GV	VDI	YNCVRELRSRRVN	MVQTEEQYVFIHDTLVEAILSKETEV		
PTP_alpha_D1	VIDAMLDMHTE	RK	VDV	YGFVSRIARQRCQ	MVQTEEQYVFIHDTLVEAILSKETEV		
PTP_opsilon_D	VIDAMMAMHAE	OK	VDV	FEFVSRIARQRCQ	MVQTEEQYVFIHDTLVEAILSKETEV		
so_CD45_D1	GIDAMLEGLEAE	GK	VDV	YGVVVKLRQRCL	MVQTEEQYVFIHDTLVEAILSKETEV		
SH_PTP2	VIDILIDIREK	GL	VDV	PKTIQMVRSQRSG	MVQTEEQYVFIHDTLVEAILSKETEV		
PTP1	VIDMLMENISTK	GL	VDV	PKTIQMVRSQRSG	MVQTEEQYVFIHDTLVEAILSKETEV		
PTP_bola	ALDRILQQLDSK	DS	VDI	YGAHVHLRLHRVH	MVQTEEQYVFIHDTLVEAILSKETEV		
s_PTP10D	TLDRILQQLDSK	DS	VDI	YGAHVHLRLHRVH	MVQTEEQYVFIHDTLVEAILSKETEV		
SAP.1	ALDVLRLQLOSE	DY	VDI	FGIVYAMRKERVW	MVQTEEQYVFIHDTLVEAILSKETEV		
PTP_STEP	ATSICCCQLRRE	GL	LGP	FSFVRKMRRESRPL	MVQTEEQYVFIHDTLVEAILSKETEV		
s_PTP69A_D1	ALDSLIIQLEEE	DS	VDI	YNTVCDLRHQRF	MVQTEEQYVFIHDTLVEAILSKETEV		
MEG2	SLDICLAQLEEL	GT	LNI	FQTVSRMRTQRNF	MVQTEEQYVFIHDTLVEAILSKETEV		
PTP_PEST	AIDYTWNLLKAG	KIPEEFNV	FNLQEMRTQRHS	AVQTKQYVFIHDTLVEAILSKETEV			
PTPH1	TMETAMCLTERN	LP	IYP	LDIVKMRDQRAM	MVQTEEQYVFIHDTLVEAILSKETEV		
PTP1	TAVIMMKLDHYFKQDYNRIDFNL	FSIVLKLREQRPG	MVQTEEQYVFIHDTLVEAILSKETEV				
ss_yeast_pyp1	VLDTLRFPESKLSGFNSVADSDV	FQVLDHIRKQRMK	MVQTEEQYVFIHDTLVEAILSKETEV				
ss_yeast_pyp2	AVDQILOVPHNLPK	TTILEDSKFEIENCNSLSRQRMK	MVQTEEQYVFIHDTLVEAILSKETEV				
n_PTP_xi_D2	VLDMLQIQIHE	GT	VNI	FGFLKHRSQRNY	LVQTEEQYVFIHDTLVEAILSKETEV		
n_LCA_D2	TLISIVLERMRYE	GV	VDM	FQTVKTLRTQRPA	MVQTEEQYVFIHDTLVEAILSKETEV		
n_PTP_alpha_D2	ALSTVLERVKAE	GI	LDV	FQTVKSLRLQRPH	MVQTEEQYVFIHDTLVEAILSKETEV		
n_PTP_opsilon_D2	ALSNILERVKAE	GL	LDV	FQAVKSLRLQRPH	MVQTEEQYVFIHDTLVEAILSKETEV		
n_PTP_mu_D2	AISIVCEMLRHQ	RT	VDV	FHAVKTLRNNKPN	MVQTEEQYVFIHDTLVEAILSKETEV		
use_CD45_D2	ALFNLLESAETE	DV	VDV	FQVVKSLRKARPG	MVQTEEQYVFIHDTLVEAILSKETEV		
os_PTP69A_D2	AMCILVQHLRLE	KC	VDI	CATTKLRSQRTG	MVQTEEQYVFIHDTLVEAILSKETEV		
n_PTP_zela_D2	ALTTLMHQLEKE	NS	VDV	YQVAKMINLMRPG	MVQTEEQYVFIHDTLVEAILSKETEV		
n_PTP_gamma_D2	ALTTLSQQLENE	NA	VDV	FQVAKMINLMRPG	MVQTEEQYVFIHDTLVEAILSKETEV		
os_PTP99A_D2	AISSIAIEMEYC	ST	ANV	YOYAKLYHNKRPG	MVQTEEQYVFIHDTLVEAILSKETEV		
rsinia_PTP	GAMCMNDNRNSQ	LSV	EDMVSQMRVORNG	MVQTEEQYVFIHDTLVEAILSKETEV			
PTBseq.no.	230	240	250	260	270	280	

PTB66

Figure 1E

a4

a5

a6

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## Vmax and Km of 37kDa-PTP1B Mutants Toward RCML

Enzyme	Vmax (nmol/min/mg)	Km (nM)	Kcat (min <sup>-1</sup> )
wild type	60200	102	2244
Tyr 46 → S	4120	1700	154
→ L	4160	1700	155
Glu 115 → A	5700	45	212
→ D	5900	20	220
Lys 116 → A	68600	150	2557
Lys 120 → A	19000	80	708
Asp 181 → A	0.61	≤126	0.023
→ E	97	10	3.6
His 214 → A	700	20	26
Cys 215 → S	0.026		0.00097
Arg 221 → K	11	80	0.41
→ M	3.3	1060	0.12
Gln 262 → A	720	9	27

Figure 2